Characterisation of novel and complex mechanisms of antobiotic resistance using a proteomics approach

Thesis

Characterisation of novel and complex mechanisms of antibiotic resistance using a proteomics approach

Tom Gaulton, BSc (Hons), MSc, PGCE

Medical Microbiology

Submitted December 2012 to the Open University for the Degree of Doctor of Philosophy

Affiliated research centre: Microbiology Services, Colindale, Health Protection Agency

Date of Submission: 26 September 2012.
Date of Award: 7 December 2012.

Contents

List of Figures

List of Tables

Acknowledgments

I would like to start by thanking each member of the supervisory team, Dr. Vesela Encheva, Dr. Raju Misra, Professor Haroun Shah and Professor Neil Woodford, for providing me with such fantastic support and advice over the course of this project.

Thanks also go to Dr. Min Fang, for the LC-MS/MS analysis of peptide extracts and general LC/MS advice and to my former colleagues Dr. Hiran Dhanji and Dr. Mike Hornsey for supplying me with the organisms for this project.

To Nabeela, whose unwavering love and support ensured that I managed to finish writing. Finally I thank my parents Jeff and Finola, to whom I owe everything. This thesis is dedicated to them.

Abbreviations used

2DGE: 2-dimensional gel electrophoresis

AMRHAI: Antimicrobial Resistance and Healthcare Associated Infections Reference Unit

ATP: Adenosine triphosphate

BSA: Bovine serum albumin

BSAC: British Society for Antimicrobial Chemotherapy

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

DIGE: difference in gel electrophoresis

DTT: Dithiothreitol

ESBL: Extended-spectrum β-lactamase

ESI: Electrospray ionisation

FDR: False discovery rate

GeLC: SDS-PAGE followed by trypsin digestion and LC-MS analysis of peptides

GTP: Guanine triphosphate

HPA: Health Protection Agency

IEF: Isoelectric focusing

IPG: Immobilised pH gradient

LB: Lysogeny broth

LC: liquid chromatography

LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry

LPS: lipopolysaccharide

MALDI-TOF: Matrix assisted laser desorption/ionisation-time of flight

MDR: Multidrug resistant

MIC: Minimum inhibitory concentration

mRNA: messenger ribonucleic acid

MRSA: Methicillin-resistant *Staphylococcus aureus*

MS: Mass spectrometry

NA: Nutrient agar

NCBI: National Centre for Biotechnology Information

NCTC: National Collection of Typed Cultures

OM: Outer membrane

OMP: Outer membrane protein

PCR: Polymerase chain reaction

PM: Phenotype microarray

PMF: Peptide mass fingerprint

PTM: Post-translational modification

RNI: Reactive nitrogen intermediates

ROS: Reactive oxygen species

rRNA: ribosomal ribonucleic acid

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

ST: Sequence type

TEMED: N,N,N',N'- tetramethylethane-1,2-diamine

TFA: Trifluoroacetic acid

tRNA: transfer ribonucleic acid

UTI: Urinary tract infection

YOUR ACCEPTANCE

1 Student details

Your full name: Tom Gaulton

Personal identifier (PI): A4045106

Affiliated Research Centre (ARC) (if applicable): Health Protection Agency, Microbiology Services - Colindale

Department: AMRHAI/DBHT

Thesis title: Characterisation of novel and complex mechanisms of antibiotic resistance using a proteomics approach

2 Authorisation statement

I confirm that I am willing for my thesis to be made available to readers by The Open University Library, and that it may be photocopied, subject to the discretion of the Librarian

Signed: ..... Tom Gaulton .....

Print name: Tom Gaulton

Date: 20/12/2012 DD/MM/YY

3 British Library Authorisation (PhD and EdD candidates only)

The Open University has agreed that a copy of your thesis can be made available on loan to the British Library Thesis Service on a voluntary basis. Please indicate your preference below:

☒ I am willing for The Open University to loan the British Library a copy of my thesis

OR

☐ I do not wish The Open University to loan the British Library a copy of my thesis

Abstract

The problem of increasing rates of antibiotic resistance has become a global concern, particularly among multidrug resistant Gram-negative nosocomial pathogens. These organisms display non-susceptibility to the majority of routinely used antibiotics, causing infections which are more difficult to treat and increase the duration of patient recovery. Due to the plethora of resistance determinants and the molecular machinery which facilitates their dissemination, new strategies are required to investigate the mechanisms that confer antibiotic resistance. Proteomic techniques allow the global analysis of the expressed proteome, providing a more holistic view of the current physiological state of the bacterial cell. The techniques used in this investigation cover the separation, quantification and identification of proteins present in cellular extracts from resistant organisms. These included the use of 2-D electrophoresis, DIGE and LC-MS/MS mass spectrometry applied to multidrug resistant *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Serratia marcescens* and *Acinetobacter baumannii.* In summary, these investigations revealed that the Tol-Pal membrane protein system and susceptibilities to polymyxin antibiotics and biocides are altered upon acquisition of a resistance plasmid in *E. coli.* Furthermore, it revealed that non-carbapenemase-mediated carbapenem resistance in *K. pneumoniae* involved the loss of fimbriae proteins, the increased expression of OmpK26 and the resistance proteins EmrA and APH(3"), in addition to OmpK35/36 porin loss. The upregulation of a multidrug efflux pump in *E. cloacae, A. baumannii* and *S. marcescens* involved the differential regulation of many proteins, spanning a broad range of functional classes, including the MinCDE cell division inhibitors, iron acquisition proteins such as FepA and FhuA and proteins involved in biofilm and LPS formation such as PapC, LptD and GmhA. Overall this project has highlighted the complex and dynamic changes in protein expression upon acquisition of a resistance phenotype and the importance of using genetically related isolates when undertaking proteomic analyses. This work also emphasised the advantages of using proteomics for profiling the expression of resistance proteins, including the detection of specific enzymes, such as CTX-M ESBLs.

1. Introduction

1.1 Antibiotics and antibiotic resistance

The implementation of modern antimicrobial therapy (1940s - post-world war II) changed the face of medicine, patient care and has had a profound impact on our society. Modern procedures rely on the use of antibiotics for surgery, organ transplants, care of premature neonates and infection management to allow successful patient rehabilitation and treatment of infections in the community. The use of antimicrobials has removed infectious disease as a top priority healthcare concern in the western world, displaced with diabetes, heart disease, and cancer. However, infectious diseases remain the leading causes of mortality in low-income countries and the third highest cause of mortality worldwide (World Health Organisation, 2011).

The term antibiotic originally described compounds naturally produced by microorganisms and which inhibited the growth of bacteria at very low concentrations (Waksman, 1972). However, the treatment of bacterial infections is hampered by resistance to antibiotics, first observed in Flemings' lab shortly after his discovery of penicillin (Fleming, 1945). Fleming himself stated: "...There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness' [resistance]...." (Fleming, 1947), noting that low levels of penicillin or short treatment cycles would induce resistance in bacteria.

The detection of resistance to antibiotics continued throughout the golden age of antibiotic discovery (said to be the 1950s; see Table 1.1) where many new drugs and drug classes were discovered to keep up with the increasing rates of resistance. For instance, the incidence of penicillin-resistant staphylococci increased throughout the 1950s until methicillin was developed and released in 1960, however methicillin-resistant *Staphylococcus aureus* (MRSA) isolates rapidly appeared within a year (Johnson 2011). After the discovery of transmissible resistance factors (R factors) in Japan (Mitsuhashi *et al.* 1960), these R factors were detected in isolates in Britain and later in Greece, with the first confirmed detection of TEM β-lactamase (Datta & Kontomichalou, 1965). Around this time, there was a surge in drug discovery, albeit most 'new' antibiotics were modified structures of existing agents *e.g.* latter generations of cephalosporins. The

evolution of resistant strains continued to catch up with these new agents at an alarming rate. Resistance is now a concern that is increasing both nationally and worldwide (Livermore *et al.* 2008; Rossolini & Mantengoli, 2008) and agents that physicians have come to rely on are being labelled as inadequate. These include antibiotics such as ampicillin and trimethoprim, successful agents which used to have widespread activity, but now *E. coli* isolates recovered from urinary tract infections (UTIs) show resistance rates of 55% and 40% for ampicillin and trimethoprim, respectively (Bean *et al.* 2008).

Class	Year discovered
Sulfonamides	1937
Penicillins	1940
Polymyxin	1947*
Chloramphenicol	1949
Tetracyclines	1953
Cephalosporins (four generations)	1953
Aminoglycosides	1957
Vancomycin	1958*
Clindamycin	1966
Rifamycin	1971
Trimethoprim/sulfamethoxazole	1973
Carbapenems	1976
Monobactams	1982
Linezolid	1987*
Daptomycin	1987*
Synercid	1992*

**Recently reintroduced*

Table 1.1. List of antimicrobials and their date of discovery. Taken from: (Davies 2006).

1.1.2 How do we define resistance?

Bacteria are principally defined as susceptible or resistant to an antibiotic based on the value of their minimum inhibitory concentration (MIC) and epidemiological cut-off value (ECOFF). An MIC is the lowest concentration of an antibiotic at which bacterial growth is inhibited, and the extent of any resistance is determined by whether it falls above or below decided concentrations or breakpoints, which vary across bacterial species and antibiotic classes. Breakpoints are decided on the basis of many factors, particularly susceptibility distribution, pharmacological properties of the antibiotic and data on the clinical outcomes of the antibiotic (Macgowan & Wise, 2001). An ECOFF is an MIC value identifying the upper limit of the wild type population for a given species and distinguishes wild-type isolates from those with reduced susceptibility. ECOFFs are determined by visual inspection of MIC histograms for a given species, or through statistical calculation (Turnidge *et al.* 2006). They are used as an indicator of resistance prevalence in surveillance studies. Breakpoint MICs are standardised by organisations such as the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews & Howe, 2011), whose guidelines are used in this thesis, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute in the US (CLSI).

1.1.3 Resistance on the rise

Resistance to multiple agents was originally commonly documented in nosocomial isolates, which is unsurprising given the selection pressures present, although there were also reports of *Shigella* isolates from dysentery patients which were resistant to streptomycin, tetracycline and chloramphenicol (Watanabe 1963). In modern times, the first probable report in 1998 of an ESBL-producing *E. coli* isolate initiated a rise to prominence (Cormican *et al.* 1998) and since then, resistance detection on community acquired infections has increased steadily (Pitout *et al.* 2005). For example, in the US in 2005, it was found that 13.7% of methicillin-resistant *Staphylococcus aureus* (MRSA) infections originated from the community (Klevens *et al.* 2007). While the general public is aware of the threat of highly publicised increases in MRSA infection, resistant Gram-

negative infections have been increasing also, but have gone somewhat unnoticed (Livermore, 2004). This problem was highlighted in a report on multiresistant *Enterobacter*iaceae isolates from the community, where many of the patients visited general practices and had no prior hospital exposure (Woodford *et al.* 2004).

Multiple studies have drawn attention to the inappropriate prescribing of antibiotics to treat infection as a contributing factor to the rise of resistance. Lai *et al.* saw links with increased prescription and resistance rates in Gram-negative bacteria, although it was antibiotic and organism-dependant. Similarly they found that resistance rates also dropped with the reduced use of certain antibiotics (Lai *et al.* 2011). Hsu *et al.* also noted similar observations, such as the increasing rates of both fluoroquinolone prescription and ciprofloxacin-resistant *E. coli*, and the association of prescription of carbapenems and imipenem-resistant *Acinetobacter baumannii* (Hsu *et al.* 2010).

1.2 Are we running out of antibiotics?

Whether appropriately prescribed or not, with the estimated production of antibiotics in the hundreds of thousands of tonnes per year worldwide (Nikaido, 2009), it is unsurprising that resistance is so widespread and frequently documented. But despite the rising rates of resistance and the critical need for novel antibiotics, the number of new agents coming on to the market is falling *e.g.* 16 agents were approved for use between 1983-87 but only seven were approved between 1998-2002 (Spellberg *et al.* 2004). Furthermore, drugs with novel modes of action, which are vital as cross-resistance to existing drugs is unlikely, are even fewer in number *e.g.* only linezolid and daptomycin had novel mechanisms of action (approved between 1998 and 2003), while the remainder were merely modified structures of existing agents (Spellberg *et al.* 2004). Lower still is the number of agents designed for use against Gram-negative pathogens, possibly due to the lower political and media attention they receive in contrast to, for example,MRSA, which is a problem well-known to the public and may have driven the focus on anti-Gram-positive agents (Theuretzbacher, 2009). This has caused a delay in the discovery and approval of new anti-Gram-

negative agents and is a cause for concern among clinicians, particularly with respect to treating ESBL-producing Gram-negative pathogens in the community (Livermore, 2009). These are among the reasons that the World Health Organisation recognises antibiotic resistance as a worldwide threat to human health (World Health Organisation, 2012).

A major problem of novel antibiotic development is that many drug companies see it as an unattractive financial risk. This is due to huge production costs for compounds which require relatively small doses and short treatment cycles to be effective and which may not have a long clinical shelf life (Kraus, 2008). Many 'Big Pharma' are focusing their efforts on compounds used in long-term treatment plans, for chronic illnesses, obesity and quality of life drugs, all of which are more likely to return greater profit than antibiotics (Kraus, 2008). However, many governments and international agencies are aware of the situation and measures are being taken to raise awareness of prescribing and misuse of antibiotics, such as the Stemming the Tide of Antibiotic Resistance (STAR) protocol, which promotes appropriate antibiotic prescription in the UK (Simpson *et al.* 2009). There is also the implementation of antibiotic stewardship programs to provide guidance to healthcare professionals on antibiotic prescribing (Charani *et al.* 2010).

The current situation of antibiotic resistance has renewed interest in antibiotic development, although the research and development of novel antibiotic compounds is increasingly being carried out in academia and smaller biotech companies rather than 'Big Pharma' (Kneller, 2010). There is also a large initiative by the Infectious Diseases Society of America (IDSA), "10 x 20" which aims to promote and sustain an R&D enterprise to develop 10 new antibacterial drugs by 2020 (Gilbert *et al.* 2010). In the UK, there is "Antibiotic Action", an initiative launched by the British Society for Antimicrobial Chemotherapy (BSAC) which has the aim of making resistance a public issue, by gathering parties from government, research, industry and charity to identify and implement solutions for the discovery and development of future antibiotics (http://antibiotic-action.com/)

1.3 Mechanisms of antibiotic resistance

There are many methods by which bacteria can become resistant to antibiotics, but the current scale of the problem and the number of resistances against drugs across different classes is unprecedented (Levy & Marshall, 2004). Listed here are a few major examples of clinically-relevant resistance mechanisms in three categories: (i) drug inactivation/modification (section 1.3.1), (ii) reduced accumulation (via reduced permeability or enhanced efflux) (section 1.3.2), (iii) target modification (section 1.3.3) and (iv) alteration of metabolic pathways (section 1.3.4). The resistances investigated in this thesis are summarised in Table 1.2, presented in Figure 1.1 and focus on Gram-negative bacteria.

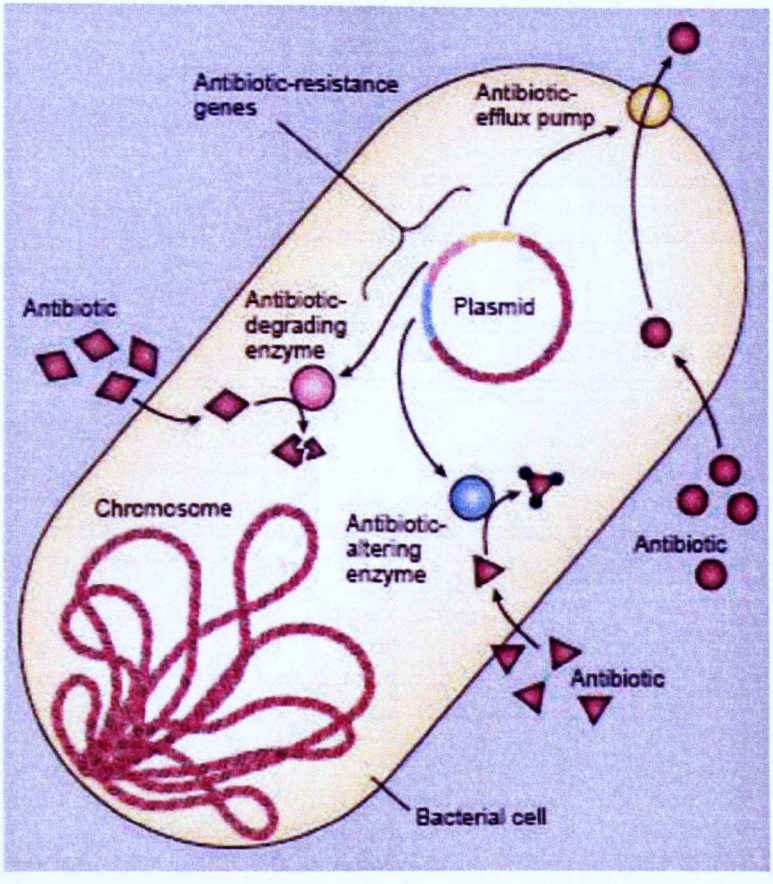

Figure 1.1 A simplified schema illustrating some of the major mechanisms of antibiotic resistance used by bacteria. Taken: from Levy & Marshall, 2004.

1.3.1 Drug inactivation

The mechanism of action of an antibiotic may be disrupted either through degradation or chemical modification of the agent, leading to changes in binding affinity and a reduction in efficacy. An obvious example is the hydrolysis of β-lactam antibiotics by β-lactamase enzymes, probably the most common resistance mechanism in Gram-negative bacteria (Bush & Jacoby, 2010). The β-lactams encompasses a vast collection of antibiotics, all with the characteristic β-lactam ring structure (Fig. 1.2). Their mechanism of action is to inhibit the enzymes involved in late stage synthesis of cell wall peptidoglycan, leading to reduced integrity of cell wall and eventually, cytosolic leakage and cell death. Paralogues (genes duplicated in the same organism) of these peptidoglycan enzymes were detected, which were able to hydrolyse and cleave the β-lactam ring, leading to loss of antibiotic activity. These enzymes are known as β-lactamases and number in the hundreds (Bush & Jacoby, 2010). Later, variants were detected that could hydrolyse more than one sub-class of β-lactam, so called extended-spectrum β-lactamases (ESBLs), see multidrug resistance, section 1.6.

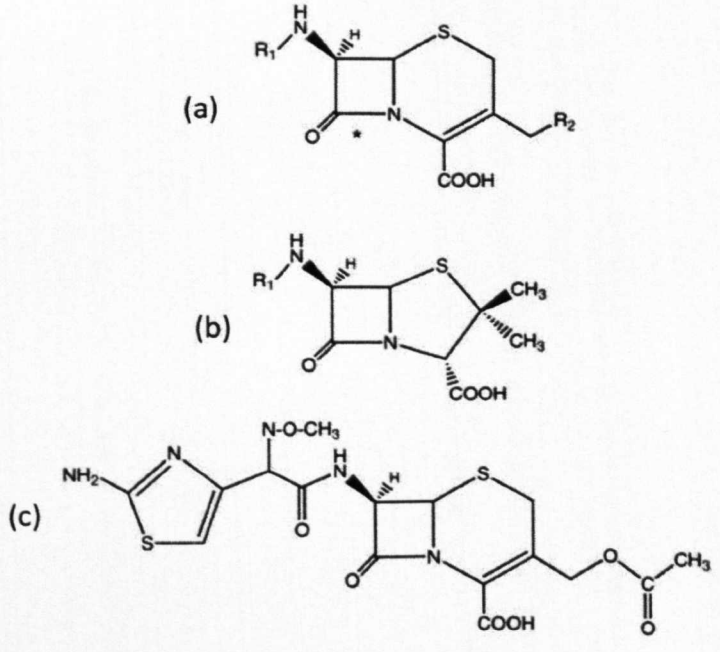

Figure 1.2 Core structures of (a) cephalosporins; (b) penicillins and structure of (c) cefotaxime. The beta-lactam ring is the square structure which simulates an amide bond. Taken from Bompard-Gilles (2000).

While many mechanisms of resistance exist for aminoglycoside antibiotics, chemical modification is by far the most prevalent (Ramirez & Tolmasky, 2011). There are multiple modifications documented which lead to the inactivation of aminoglycosides, through modification of their hydroxyl and amino groups, which are required to interact with the ribosomal machinery. The modifications which mediate aminoglycoside inactivation include N-acetylation *e.g.* the enzyme AAC(6')-Ib, phosphorylation *e.g.* APH(3') and adenylation *e.g.* ANT(6) (Ramirez & Tolmasky, 2011). Each enzyme has a specific spectrum of activity and as many of these enzymes are plasmid-encoded, organisms have been documented carrying multiple aminoglycoside-modifying enzymes (Davies & Wright, 1997).

1.3.2 Reduced drug accumulation

An obvious way to reduce the accumulation of an antibiotic is to prevent entry to the cell altogether. By reducing the expression of the outer membrane porins, cells can restrict the entry of a variety of compounds. Porins are outer membrane proteins which cross the outer membrane and act like a channel through which molecules may pass *e.g.* metabolites (in) or toxins (out) (Martínez-Martínez, 2008). As porins have different sized pores, bacteria can prevent entry of antibiotic molecules while still allowing other nutrients free passage across the outer membrane through selective porin regulation. For instance this can occur in *E. coli*, through reduction of OmpC or OmpF expression while not reducing OmpA (Tenover, 2006). This is a common strategy for bacteria, particularly against carbapenem antibiotics, and has been documented in *Pseudomonas aeruginosa, Enterobacter cloacae* and *Klebsiella pneumoniae* (Doumith *et al.* 2009; Jacoby *et al.* 2004).

Mechanism	Target antibiotic class	Example	Outcome	Relevant organisms	References
Antibiotic modification/inactivation	β-lactam antibiotics *e.g.* penicillin, cephalosporins and carbapenems	β-lactamases, ESBLs, carbapenemases	β-lactam ring of the antibiotic is hydrolysed and opened, antibiotic activity is lost	Members of the *Enterobacter*iaceae	Canton 2012, Jacoby 2009, Bush 2010
Reduced antibiotic accumulation	vast range of target molecules including: antibiotics, biocides, heavy metals	efflux pumps *e.g.* AcrAB-TolC in many *Enterobacter*iaceae	while antibiotics enter the cell, they are quickly removed and do not accumulate to active levels	expressed across many species of bacteria	Piddock 2006, Martinez 2009
	e.g. β-lactams, fluoroquinolones, tetracycline	reduced permeability *e.g.* reduced expression of porins, OmpC/OmpF in *E. coli*	reduced porin expression reduces the molecular entrances to the cell, resulting in insufficient accumulation of antibiotic levels	Members of the *Enterobacter*iaceae	Martinez-martinez 2008, Doumith 2009
Antibiotic target modification	*e.g.* targets of daptomycin, fluoroquinolones,	*e.g.* lysylation of phosphatidylglycerol in the cell envelope, mutations in DNA gyrase enzymes	Antibiotic can no longer bind to its highly specific target site and is rendered inactive	*S. aureus* for daptomycin,	Friedman 2006, Jacoby 2005
Alteration of metabolic pathways	targets of sulphonamides and trimethoprim	replacement of DHTS and DHFR in the folate metabolic pathway	replacement of susceptible enzyme with resistant one renders antibiotic inactive	*E. coli*, *Shigella,*	Wise 1975, Skold 2001

Table 1.2 Summary of main mechanism of antibiotic resistance discussed in section 1.3.

An alternative method of reducing antibiotic accumulation is to increase the efflux of antibiotics from the cell. Efflux as a mechanism for antibiotic resistance was first described by Mcmurry *et al.* (Mcmurry *et al.* 1980) who demonstrated that efflux pumps were responsible for tetracycline resistance. Now the view is that efflux pumps are widely distributed across all domains of life and serve a variety of functions in the life cycle of bacteria to interact with their environment (Martinez *et al.* 2009). There are many types of efflux pump, three of which confer multidrug resistance (MDR; see section 1.6) phenotypes in Gram-negative bacteria: the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily and the resistance-nodulation-cell division (RND) superfamily (Piddock, 2006). Here the focus will be on the RND-type family as these are the pumps most-often associated with resistances of clinical significance. For instance, they include: AcrAB-TolC, present in many *Enterobacter*iaceae such as *E. coli*, *Salmonella typhimurium* and *Enterobacter cloacae* (Pérez *et al.* 2012). The MexXY-OprM and MexAB-OprM pumps in *P. aeruginosa,* which provide inherent non-susceptibility to many compounds, including fluoroquinolones, tetracyclines, aminoglycosides, macrolides and chloramphenicol (Tenover, 2006), also the AdeAB-TolC pump in *Acinetobacter baumannii* (Hornsey *et al.* 2010a). These RND pumps consist of three subunits, in the case of AcrAB-TolC; AcrB acts as the transporter or efflux protein, located on the cytoplasmic membrane. AcrA is the periplasmic linker subunit (known as a membrane-fusion protein) which spans the periplasmic space. In this pump TolC is the outer membrane protein (OMP) channel used to extrude the molecule, although many proteins may be used as OMP channels depending on the specific pump. AcrABC binds substrates from the inner membrane or the cytoplasm and transports them to the extracellular medium (Piddock, 2006).

1.3.3 Target modification

Antibiotic targets may be modified either through natural chromosomal mutation or changes which lead to chemical modification by host enzymes. Examples of antibiotic targets which mediate resistance through modification include daptomycin, an anti-Gram-positive agent which was the first in the novel class of lipopeptides. Its mechanism of action, though not entirely understood, involves binding and subsequent calcium-dependent insertion into the bacterial cell wall. This

disrupts the cell membrane, causing ion efflux, closely followed by loss of the ion concentration gradient and depolarisation of the membrane, disrupting macromolecule synthesis and leading to cell death (Beiras-Fernandez *et al.* 2010). There are many potential genes in which mutations could give rise to daptomycin non-susceptibility, including *mprF*, *yycG*, *rpoB* and *rpoC* (Friedman *et al.* 2006). The association of MprF with daptomycin resistance has been well documented in *S. aureus* and is involved in the synthesis of the phospholipid precursor phosphatidylglycerol (Oku *et al.* 2004, Kristian *et al.* 2003). MprF catalyses the transfer of a lysine residue onto phosphatidylglycerol to yield lysylphosphatidylglycerol, the incorporation of this into the membrane increases the overall positive charge and repels Ca^{2+} ions, which are required for daptomycin activity, rendering the organism resistant (Thedieck *et al.* 2006). This resistance mechanism also confers resistance to a variety of cationic antimicrobial peptides, found both in the environment *e.g.* soil, and in mammalian hosts as part of the immune system. This resistance mechanism may confer a more virulent phenotype and increase the difficulty of organism clearance during treatment.

Target modification is one method of resistance utilised against the fluoroquinolone antibiotics. Although the accumulation of mutations in the target proteins are most common (Poirel *et al.* 2012), other methods include reduced accumulation and plasmid-encoded proteins which prevent quinolone activity. These agents act on the essential bacterial enzymes DNA gyrase (primary target in Gram-negative bacteria) and DNA topoisomerase IV (primary target of Gram-positive bacteria), large complex enzymes involved in the positive and negative supercoiling of DNA. This is an important process in all aspects of nucleic acid maintenance and metabolism including the replication, transcription, recombination, and repair of DNA (Jacoby, 2005). The enzymes also have endonuclease properties, required to break double stranded DNA (dsDNA) and ligate it with incorporated supercoils. The quinolones exploit this activity and trap the enzymes when bound to dsDNA, causing unchecked breaks in the DNA to occur, leading to cell death. Mutations in the DNA-binding domain of the genes encoding gyrase (*gyrA*) and topoisomerase IV (*parC*) have been shown to decrease the binding affinity of quinolones, inhibiting their activity and conferring resistance (Piddock, 2002).

1.3.4 Alteration of metabolic pathway

The alteration of a metabolic pathway (or metabolic bypass) to confer resistance to an antibiotic involves acquiring a novel variant of an antibiotic-susceptible enzyme and using it in place of the existing enzyme, 'bypassing' the metabolic susceptibility. This process can involve the addition of whole operons rather than just single gene transfer. Examples include sulphonamide resistance: the sulphonamides, while not the first antimicrobials discovered, were the first to be used in large scale treatment of infections. They act upon the essential tetrahydrofolate biosynthetic pathway, specifically they are competitive inhibitors of dihydropteroate synthase (DHPS), which starves the bacteria of folate and leads to eventual cell death. Bacteria have circumvented the effects of this agent by replacing DHPS with a sulphonamide-resistant variant, either *sulI* or *sulII*, effectively bypassing the enzyme susceptible to antibiotic action (Wise & Abou-donia, 1975). There are few available variants due to the constraints imposed upon the enzyme to retain normal activity as well as sulphonamide resistance. However, due to their efficient vehicles for dissemination, such as the transfer and acquisition of resistance plasmids (see section 1.5), these enzymes are widespread and account for the vast majority of sulphonamide resistance in Gram-negative bacteria (Sköld, 2001). A similar mechanism is employed for resistance to trimethoprim, another synthetic antibiotic which acts on the same pathway as the sulphonamides, but on the enzyme dihydrofolate reductase (DHFR), which inhibits the synthesis of tetrahydrofolate. Plasmid-encoded *dfr* genes express trimethoprim-resistant forms of the enzyme to confer resistance to trimethoprim and unlike the sulphonamides, there are numerous forms of the enzyme (Sköld, 2001).

The best characterised example is probably recruitment of the *mecA* gene by methicillin-resistant *Staphylococcus aureus* (MRSA). *S. aureus* has a variety of penicillin-binding proteins (PBPs) involved in peptidoglycan turnover of the cell wall *e.g.* PBP2 has transpeptidase and transglycosylase activities. Normally, the presence of β-lactams would inhibit the PBPs and lead to eventual cell death due to loss of cell wall integrity. However, PBP2a encoded by the *mecA* gene and transported on a mobile genetic element called the staphylococcal cassette chromosome (SCCmec) is not susceptible to most β-lactams and provides transpeptidase activity unhindered (Fuda *et al.* 2005). The result is that MRSA isolates have resistance to most β-lactam antibiotics.

1.4 Physiological role of resistance components: resistance in the environment

Given the number of resistances across different drug classes and the speed with which bacteria acquire resistance to agents with novel mechanisms of action, many investigators have turned to the environment to seek out sources of resistance or reservoirs of resistance mechanisms. Indeed, there are obvious animal sources of drug resistance, such as from the farming of animals for meat and poultry. Here antibiotics were routinely used (and still are in developing countries) as growth promoters to produce much larger animals, allowing the energy and nutrient resources previously used to fight off infections to be used to increase animal growth and prevent loss of livestock due to illness (Dibner & Richards, 2005). Antibiotics are also heavily used in agriculture, administration of antibiotics can boost product yield by preventing disease and pests although this is a practice which is diminishing *e.g.* in 2006 the EU placed a ban on the feeding of all antibiotics to farm animals for growth purposes (upgraded from a partial ban placed in 1998) (Martinez, 2009).

For a time it was believed that these human-influenced environmental sources of antibiotics were solely responsible for the rise in resistance, however mechanisms of resistance have been discovered in the natural environment far away from human-populated areas. For example, *E. coli* resistant to tetracyclines, ampicillin, chloramphenicol and streptomycin were detected in 92% of the remote, high-altitude community of Peruvian Amazonas, far-removed from modern antibiotic exposure (Bartoloni *et al.* 2009). There have also been reports of bacteria from the pre-antibiotic era possessing resistance enzymes *e.g.* Song *et al.* recovered bacteria from deep ocean sediments (c. 10,000 years old) and found that a small number carried ESBLs highly similar to TEM ESBL (Song *et al.* 2005). D'Costa and colleagues reported on the detection of resistance genes in bacteria sampled from ice cores over 30,000 years old and detected, among others, aminoglycoside resistance protein AAC(3), tetracycline protection protein TetM, a member of the TEM β-lactamases and VanX, a component from the vancomycin resistance operon (D'Costa *et al.* 2011).

Hence, resistance to antibiotics, and the dissemination of resistance, is not a novel phenomenon. As Allen *et al.* discuss, resistance genes in the natural environment are extremely prevalent (Allen *et*

al. 2010). Enzymes conferring drug resistance often have natural roles in bacteria, some are variants of proteins possessing essential functions in cellular physiology *e.g.* penicillin-binding proteins were originally involved in the maintenance and modification of cell wall peptidoglycan. Furthermore, CTX-M β-lactamases originated from a variant of penicillin-binding proteins in *Kluyvera* sp. (Cantón *et al.* 2012) and TetX genes found to confer tetracycline resistance in soil-dwelling *Bacteroides fragilis*, were originally used as monooxygenase enzymes (Volkers *et al.* 2011). Efflux pumps are involved in many natural processes, from reduced accumulation of toxic compounds from environment, such as heavy metals, to the extrusion of signal molecules mediating cellular communication (Martinez *et al.* 2009).

There is an abundant natural source of potential resistance genes providing a repository from which pathogenic bacteria could draw on. This was recently highlighted by D'Costa, who showed that even for daptomycin, one of the few novel classes of antibiotic for years, multiple resistance mechanisms were found in environmental actinomycetes, including ring hydrolysis and acetylation of the hydrophobic tail (D'Costa *et al.* 2012). As we come to understand more of the microbial biosphere, it seems that the reservoir of resistance that many are searching for may be bacteria themselves (Forsberg *et al.* 2012). While this may answer how bacteria may rapidly become resistant to even novel agents without requiring previous exposure, it does not explain how specific- and multidrug-resistances have become so widespread.

1.5 Molecular spread of resistance

The most obvious method of resistance transmission would be to pass on the gene directly from mother cell to daughter cell *i.e.* the vertical spread of resistance. An increase in the prevalence of a resistance mechanism is often aided by the expansion of successful bacterial lineages or clones (descendants of a common strain). Examples of successful clones include ST131 *E. coli,* which often carry CTX-M-15, ST258 *Klebsiella pneumoniae*, which often carry KPC carbapenemase and the OXA-23 clone 1 *Acinetobacter baumannii* (Woodford *et al.* 2011).

As described in section 1.4, resistance to a variety of antibiotics has existed in the environment for millennia, it is likely that the means to spread these resistance determinants

between bacteria have been around just as long *e.g.* horizontal gene transfer (HGT). The ease of resistance transmission combined with successful clones, can result in the rapid spread of a resistance mechanism. There are three main molecular mechanisms of HGT and resistance dissemination in bacteria: bacteriophages; transposable genetic elements; and plasmids (see Fig. 1.3); (i) bacteriophages are bacterial viruses which infect prokaryotic cells and deliver their genetic material from a protein capsid. These genes code for proteins to make more viruses, utilising the host's replication machinery to copy their DNA and express capsid proteins to make more phage particles (Frost *et al.* 2005). When packing the phage particle with DNA, bacterial chromosomal material adjacent to the phage may be excised also, packaged with the prophage and upon infecting a new host, integrated it into the new chromosome. Sometimes there may be no phage genes at all and the defective virion serves only to transfer bacterial DNA from one cell to another (Brussow *et al.* 2004). Bacteriophages are important for bacterial pathogenesis, pathogens such as *Corynebacterium diptheriae*, *Clostridium botulinum* and *Streptococcus pyogenes* all contain phage-encoded toxins (Brussow *et al.* 2004) and are also known to play a role in the dissemination of antibiotic resistance genes (Fancello *et al.* 2011).

(ii) Transposable genetic elements, including: insertion sequences (ISs), transposons and integrons within. The transposases (enzymes which carry out the insertion and excision activities) encoded by transposons and ISs are believed to be the most abundant proteins in nature (Aziz *et al.* 2010), highlighting the fundamental role this widely used process plays in the evolution and ecology of all forms of life.

ISs are the simplest transposable genetic elements, they consist of only genes encoding transposition activity, usually flanked by inverted repeat sequences and do not carry other accessory genes *e.g.* antibiotic resistance genes. They are able to insert themselves into a DNA molecule through the transposase, which has the potential to cause mutations *e.g.* insertional inactivation, and promotes bacterial genetic diversity (Toleman & Walsh, 2011). ISs are frequently found on plasmids and are often linked with antibiotic resistance genes *e.g.* IS*Ecp1* and IS*CR1* enable the mobilisation of $bla_{CTX\text{-}M}$ genes (Cantón & Coque, 2006).

Transposons are similar to IS in that they contain a transposase flanked by inverted repeats, however some transposons also carry a repressor to regulate transposition. They regularly carry

accessory genes between the inverted repeat regions and transfer them to other locations on the chromosome or to different cells (Frost *et al.* 2005).

Integrons are based on a platform that incorporates genes by site-specific integration and all contain three basic elements: the *intI* gene, encoding an integrase; a promoter Pc and a specific recombination site *attI*, which allows the recombination of various resistance gene cassettes, encoding resistance determinants to almost every type of antibiotic (Stalder *et al.* 2012).

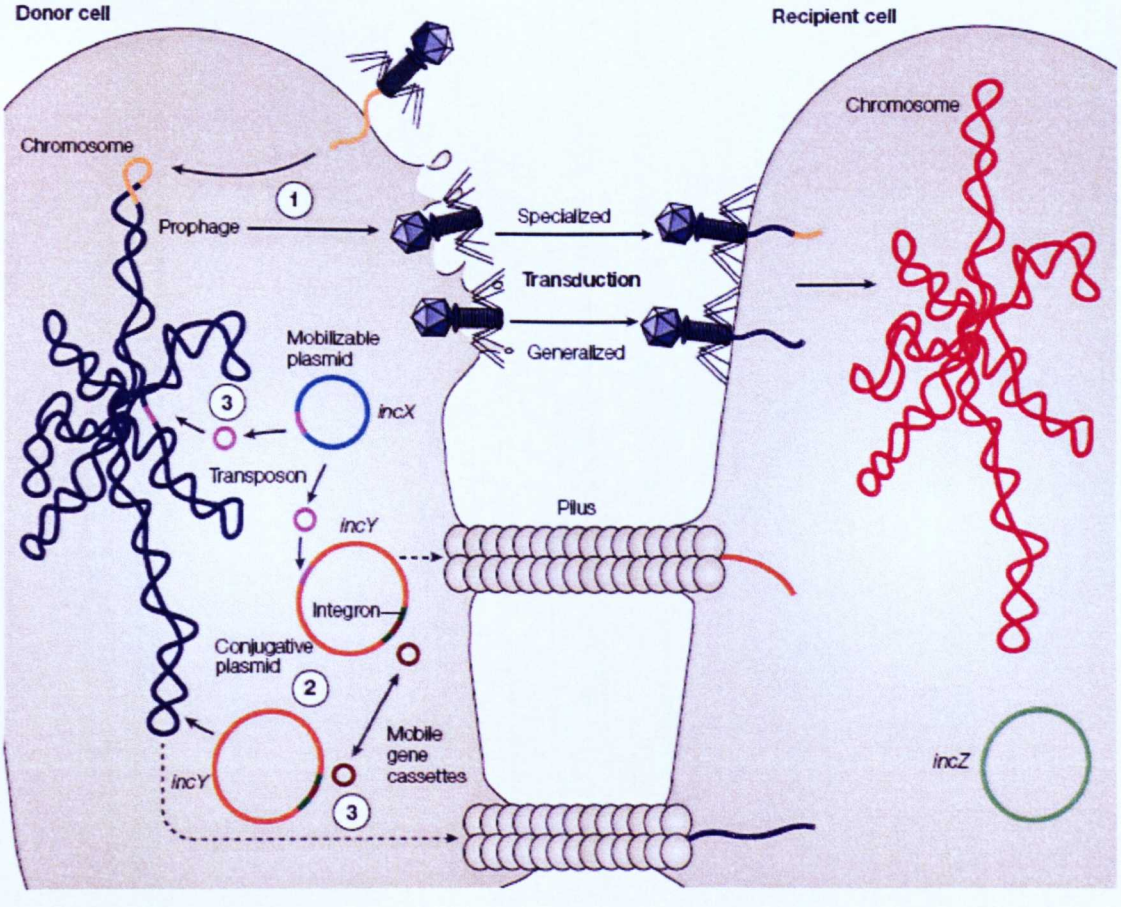

Fig. 1.3 A schema describing the potential molecular mechanisms which promote dissemination of resistance genes. 1) Transduction 2) Conjugation 3) Transposition. Taken from: Frost (2005)

(iii) Plasmids are (usually) circular molecules of DNA that can replicate independently from the chromosome and provide extra genes, which may provide an advantage to the host *e.g.* they may code for toxins, metabolic proteins and antibiotic resistance determinants. Plasmids differ from the previously mentioned elements in that they may carry many genes and it is quite usual to see resistance plasmids of more than 100 kb (Karisik *et al.* 2006). They may be either conjugative, that is, encoding the proteins required to facilitate cell-to-cell DNA transfer and can have a range of hosts, or they may be non-conjugative, that is, they do not have the machinery for cell-to-cell transfer but still contain enzymes to transfer individual genes (Bennett, 2008). In Gram-negative bacteria, this machinery is known as a sex pilus such as the F pilus of *E. coli*, similar to type IV secretion apparatus which acts to pull two bacterial cells together, fuse their membranes and allow exchange of cytoplasmic material (Bennett, 2008). Conjugation has played an important role in the dissemination of resistance genes between and within many bacterial pathogens, as there is minimal energy cost to the host, replication is host-independent and no recombination is necessary for genetic insertion (Carattoli, 2009).

1.6 Multi-drug resistance (MDR)

Given the variety of potential mechanisms that exist for antibiotic resistance, and the fact that the genetic determinants for these resistance mechanisms are able to mobilise and transfer freely from one organism to another, it is unsurprising that sometimes organisms pick up more than one. Most of the previously mentioned mechanisms of antibiotic resistance (section 1.3) were single determinants of resistance, allowing tolerance of one particular compound or group of compounds. However, combinations of these mechanisms (or alone for some mechanisms *e.g.* drug efflux) can lead to multidrug resistance or MDR. An MDR organism is defined as one that is simultaneously resistant to several (more than three) structurally and functionally different drugs (Magiorakos *et al.* 2011). It was originally described by Watanabe referring to strains of *Shigella dysenteriae* which harboured transferable resistance determinants for streptomycin, tetracycline and sulphonamides in Japan (Watanabe, 1963).

MDR organisms are resistant to agents crossing different drug classes, possibly without the need for prior exposure, and in some cases can spread intra- and inter-specially with great rapidity due to the aforementioned molecular mechanisms promoting dissemination (Cantón & Coque 2006; Smillie *et al.* 2010). Combined with the numerous molecular methods of resistance dissemination, the spread of MDR bacteria is a key threat to global health and the reason for this study.

Mechanisms of resistance that confer MDR phenotypes include: (i) Efflux pumps, which were mentioned previously in section 1.3.2, but given that RND efflux pumps can extrude many different chemicals and chemical classes non-specifically, it follows that this molecular mechanism is capable of conferring MDR to a variety of antibiotics. Although efflux pumps do not naturally confer MDR phenotypes, a simple mutation in a promoter/repressor to upregulate the pumps, may lead to MDR (Keeney *et al.* 2007; Hornsey *et al.* 2010a; Ruzin *et al.* 2005). An example of an efflux pumps conferring MDR has been demonstrated by Hornsey *et al.* who showed that treatment of *E. cloacae* with ciprofloxacin caused upregulation of the AcrAB-TolC pump, conferring resistance to ciprofloxacin and also tigecycline (Hornsey *et al.* 2010b).

(ii) The term extended-spectrum β-lactamases (ESBLs) was first coined to describe variants of the TEM and SHV β-lactamases. The criteria were: (i) high catalytic rates for the oxyimino-cephalosporins and (ii) extended spectrums of activity compared to their parent enzymes (Livermore, 2008). The term now includes many enzymes which only have to meet either one of the criteria (Livermore, 2008). There are now multiple classes of ESBL with hundreds if not thousands of members *e.g.* Class A (CTX-M ESBL), Class B (metallo-β-lactamases), Class C (AmpC ESBL) and Class D (OXA ESBL).

Class B includes many of the carbapenemase enzymes, which represent the most versatile family of β-lactamases and one with the greatest spectrum of activity. Although called carbapenemases, many of these enzymes have activity against the majority of β-lactams but without the susceptibility to β-lactamase inhibitors *e.g.* clavulanic acid (Queenan & Bush, 2007). The class B enzymes contain zinc metal in their catalytic sites rather than serine, which is utilised by other clinically problematic carbapenemases including KPC, originally from *Klebsiella*

pneumoniae, and the OXA carbapenemases, a problem commonly associated with *Acinetobacter baumannii* (Woodford *et al.* 2006). More recently NDM-1 was discovered, amid much controversy, and was found to confer resistance to almost all available beta lactam antibiotics, including the clinically important carbapenems. As it has the potential to disseminate in a similar manner to the CTX-M and carbapenemase enzymes before it, it represents a global threat in the treatment of MDR infections (Kumarasamy *et al.* 2010).

(iii) These mechanisms may be exacerbated when combined with porin deficiency, as it widens the resistance profile of the organism. For example, chromosomal AmpC in combination with porin deficiency in *Enterobacter cloacae* gives resistance to 4th generation cephalosporins (Paterson, 2006). ESBL-producing *K. pneumoniae* with reduced porin expression can potentially be resistant to carbapenems (Martínez-Martínez, 2008). It is thought that although ESBLs have poor rates of hydrolysis of carbapenems, the reduced accumulation conferred by alterations in porin expression allows the degradation of carbapenems at low levels (Nikaido, 2009).

(iv) As mentioned previously in section 1.5, resistance plasmids are capable of carrying many genes (>100) and can be very large (>100 kb). Examples of MDR plasmids include incompatibility group IncF, which often carry the $bla_{CTX\text{-}M}$ genes e.g the $bla_{CTX\text{-}M\text{-}15}$ gene is often associated with $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$ and *aac(6')-Ib-cr* genes (Carattoli, 2009). Today there are plasmids harbouring multiple resistance determinants covering different classes, capable of conferring MDR with just one plasmid transfer. This contributes heavily to explain why bacterial pathogens whose treatment was once straightforward are now resistant to many or all of the treatment options (Rossolini & Mantengoli, 2008). This is exemplified by the rise of MDR Gram-negative pathogens resistant to all routinely-prescribed agents, which are an increasing concern (Livermore, 2004). The organisms selected for investigation in this thesis are all MDR Gram-negative pathogens and represent resistances of public health concern.

1.7 Resistances investigated in this thesis

1.7.1 ESBL-producing *Escherichia coli*

Extended-spectrum β-lactamases (ESBLs) hydrolyze a wider range of β-lactam antibiotics and were first reported in the 1980s as variants of the classical SHV and TEM β-lactamases. CTX-M enzymes have subsequently established themselves as the dominant ESBLs worldwide (Bonnet, 2004; Cantón & Coque, 2006). Their name is derived from their ability to hydrolyse cefotaxime (CTX) and they are defined by a weaker activity against the related compound ceftazidime (Woodford *et al.* 2004). CTX-M-15 is the most successful CTX-M ESBL in the UK and globally, it has a greater activity against ceftazidime when compared to most CTX-M enzymes (Poirel, 2002). The CTX-M ESBL-encoding genes are thought to have been captured from the chromosome of *Kluyvera* spp. on conjugative plasmids, which helped mediate their dissemination (Cantón & Coque, 2006; Rossolini & Mantengoli, 2008).

CTX-M-15 ESBL is usually encoded on large multi-resistance plasmids, with *E. coli* now the main host species (Cantón & Coque, 2006). This is a problem in itself as *E. coli* is a highly prevalent organism and the most frequent causative agent of bacteraemia and urinary tract infections (UTIs) (Livermore *et al.* 2008). These plasmids not only facilitate the spread of ESBLs but they can also harbour a range of unrelated antibiotic resistance genes, seriously reducing the treatment options of plasmid-containing organisms. For example, the plasmids found in successful UK clones contain genes for resistance to trimethoprim, tetracycline, chloramphenicol and aminoglycosides (Bonnet, 2004; Karisik *et al.* 2006). These additional resistances also aid the spread of CTX-M ESBLs via indirect selective antibiotic pressure *i.e.* selection pressure with an antibiotic on the same plasmid as a CTX-M enzyme could select for CTX-M-producing strains even though the organism was never exposed to cefotaxime.

Different strains harbouring defined resistance plasmids/genes are endemic in countries across the world (Cantón & Coque, 2006). The CTX-M enzymes are thought to be so widely disseminated in part due to the spread of pandemic uropathogenic clones and partly because of the accumulation of highly transmissible genetic elements on successful plasmids. These elements

include insertion sequences, transposons and integrons, some of which act as promoters for the resistance genes themselves (Dhanji *et al.* 2011).

1.7.1.2 *Escherichia coli*

Escherichia coli is a natural commensal Gram-negative organism and is one of the most extensively studied bacterial species. However, some strains have diverged to form pathogenic variants, able to cause disease within the intestine (diarrheagenic *E. coli*) or outside it (extraintestinal pathogenic *E. coli* or ExPEC). These pathogenic strains have subtypes or pathotypes, possessing similar virulence factors and displaying similar disease outcomes (Wiles *et al.* 2008). The pathotypes have distinct sites of infection, but one of the most commonly encountered is uropathogenic *E. coli* (UPEC). Although they are extraintestinally pathogenic, UPEC isolates still reside in the gut, which is thought to be a reservoir for these pathogens (Sivick & Mobley, 2010). About 70-95% of community urinary tract infections (UTIs) and about 50% of nosocomial UTIs involve UPEC isolates, which often involve recurring infections (Wiles *et al.* 2008). It is known that specific clonal isolates of *E. coli* have participated in the global dissemination of CTX-M enzymes, such as ST131 which is associated with CTX-M-15 carriage. Also, the majority of *E. coli* which make up these pathogenic clones are UPEC isolates (Cantón *et al.* 2012). As UTIs are one of the most common human infections, it becomes clear that this is a model for resistance dissemination which is worth investigating to probe ways of preventing global dissemination of resistance determinants. Although the resistance determinants on the plasmids used in this study have been well characterised previously (Dhanji *et al.* 2011; Karisik *et al.* 2007), this study utilises proteomics to probe deeper into the physiological changes in the cell.

1.7.2 Carbapenem resistant *Klebsiella pneumoniae*

1.7.2.1 Carbapenems and carbapenemases

Carbapenems have the broadest activity of all β-lactam antibiotics and are often the agents of last resort to treat serious infections caused by Gram-negative bacteria (Kattan *et al.* 2008). The

carbapenems are a relatively recent addition to the antibacterial arsenal; imipenem and meropenem were the first agents to be released (1984 and 1995, respectively) and resistance has been sparse. However, when ertapenem, a new agent was licensed in 2001, cases of resistance were reported relatively rapidly as ertapenem has decreased activity as compared with other carbapenems against bacteria that produce ESBLs (Kattan *et al.* 2008). There have also been cases where treatment of an infection with imipenem has actually selected for ertapenem resistance (Lartigue *et al.* 2007). Although as cell impermeability was a factor, this may just have been class-wide cross-resistance.

More recently, production of a carbapenemase has become one of the main mechanisms of resistance to carbapenems. Most of these diverse enzymes have an extremely broad substrate range and are able to hydrolyse many β-lactam antibiotics (Kattan *et al.* 2008). At the same time, many are unaffected by β-lactamase inhibitors (Queenan & Bush, 2007). Other mechanisms of carbapenem resistance have been described, such as reduced membrane permeability (often through reduced expression of outer membrane porins), up-regulated efflux pumps (which expel the antibiotic *e.g.* ertapenem, from its site of action) or a combination of mechanisms (Szabó *et al.* 2006). For instance, *E. coli* isolates that hyperproduce an AmpC β-lactamase or ESBL and also have decreased permeability through down-regulated expression of outer membrane proteins (OMPs; *e.g.* OmpC/OmpF), are resistant to ertapenem (Mammeri *et al.* 2008; Poirel *et al.* 2004).

While there is concern regarding the wider dissemination of carbapenemases, the genes encoding them can be detected by PCR. However, non-carbapenemase-producing isolates pose problems for reference laboratories, where isolates such as *K. pneumoniae* present high carbapenem MICs but are negative for any carbapenemase (Woodford *et al.* 2007).

1.7.2.2 *Klebsiella pneumoniae*

K. pneumoniae is a commensal organism and is also present in the environment, it is however an opportunistic, Gram-negative pathogen capable of causing severe disease in humans and animals. It is a prominent nosocomial pathogen that frequently causes respiratory infections, bacteraemia and UTIs (Brisse *et al.* 2009). Carbapenems are one of the last remaining treatment options for MDR

ESBL-producing *K. pneumoniae*. Now carbapenem-resistant *K. pneumoniae* are on the rise worldwide, with endemic situations in some countries (Nordmann *et al.* 2009). As with other MDR *Enterobacter*iaceae isolates, there are high mortality rates (c. 40%) associated with this organism and debilitated patients with prior antibiotic treatment are at the greatest risk (Nordmann *et al.* 2009).

While much attention has been given to carbapenemase-producing *Enterobacter*iaceae, the combination of reduced permeability and ESBL production still occurs (Webster *et al.* 2010) and can give erroneous results in antibiotic resistance assays *e.g.* a non-carbapenemase-producing isolate with reduced porin expression and an ESBL would give MICs similar to carbapenemase producers. For example in a recent study conducted in Chile, 61 carbapenem resistant *Enterobacter*iaceae isolates were tested for carbapenemase and all returned as negative (Wozniak *et al.* 2012). For non-carbapenemase-producing *K. pneumoniae,* it was found that porin alteration was the most important factor in carbapenem resistance rather than presence of an ESBL (Wozniak *et al.* 2012).

Although the underlying changes resulting from this resistance mechanism are poorly defined, it is probable that they will involve changes to the bacterial proteome. Utilising a proteomic approach may help to elucidate these changes and the potential causes of them such as direct changes in the genes, or their regulatory sequences or indirectly through changes in global regulatory loci. For instance changes in the *mar* locus of *E. coli* can produce a multidrug resistance (MDR) phenotype via up-regulation of the AcrAB-TolC tripartite efflux system (Alekshun & Levy, 2007; Randall & Woodward, 2002). The carbapenems have been referred to previously as agents of last resort or 'silver bullets', but with resistance on the rise, there is the potential that carbapenems may be rendered ineffective in the future. Therefore, investigation into these mechanisms of resistance is of high importance to public health.

Using proteomics to analyse the changes in protein expression profile between carbapenem-susceptible and -resistant non-carbapenemase-producing isolates may elucidate other proteins which have a role in the resistance mechanism, a protein marker for reduced porin expression or even a protein which may serve as a novel antimicrobial target.

1.7.3 Tigecycline resistance

Tigecycline is a semi-synthetic derivative of minocycline and is a member of the novel glycylcycline class of antibiotics, based on the tetracycline molecular frame (Fig 1.4) (Kelesidis *et al.* 2008). Tigecycline retains activity against isolates carrying the *tet* genes encoding tetracycline efflux and ribosomal-protection resistance mechanisms (Fritsche *et al.* 2005). Tigecycline has a broad range of *in vitro* activity across many Gram positive species such as MRSA (Fritsche *et al.* 2005) and anaerobes such as *Bacteroides*, *Prevotella* and *Clostridium* sp. (Nagy & Dowzicky, 2010).

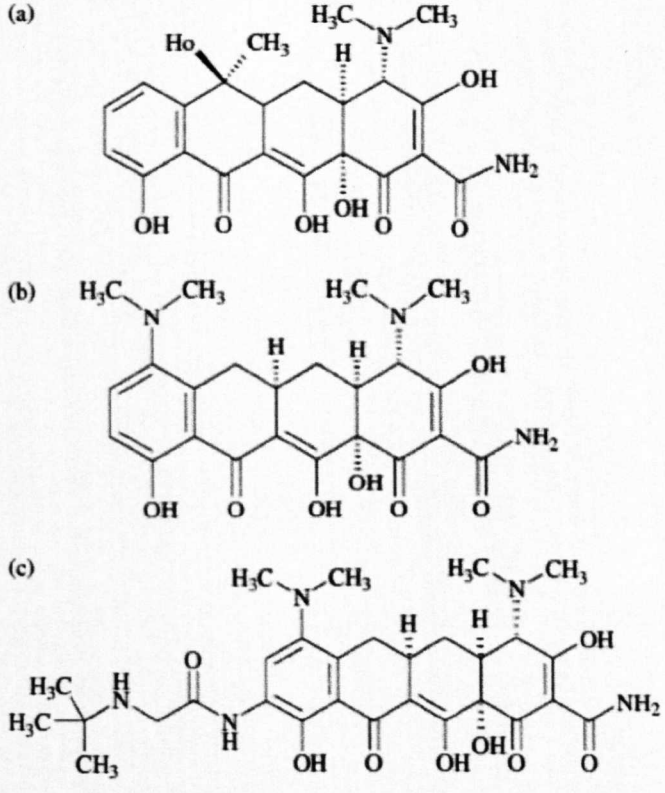

Figure 1.4 Chemical structures of a) tetracycline b) minocycline and c) tigecycline. Taken from Livermore *et al.* (2005).

Tigecycline also had *in vitro* activity against many members of the *Enterobacter*iaceae and particularly against ESBL-positive MDR *E. coli* and *Klebsiella* sp. isolates (Kelesidis *et al.* 2008) and also MDR and non-MDR *A. baumannii* (Karageorgopoulos *et al.* 2008). However, *Pseudomonas aeruginosa* and the *Proteae* are inherently resistant due to the possession of multiple RND efflux pumps (Livermore, 2005). This same resistance mechanism is also possible in other Gram-negative pathogens, the upregulation of RND efflux pumps as a mechanism for tigecycline resistance has been documented in organisms such as *Klebsiella pneumoniae* (Ruzin *et al.* 2005), *Acinetobacter baumannii* (Hornsey *et al.* 2010a; Peleg *et al.* 2007), *Enterobacter cloacae* (Hornsey *et al.* 2010b; Keeney *et al.* 2007) and *Serratia marcescens* (Hornsey *et al.* 2010c).

There are very few agents to which tigecycline-resistant MDR pathogens remain susceptible, often the last antibiotics available for tigecycline-resistant organisms are the polymyxins (*e.g.* colistin and polymyxin B). These agents were never widely used due to their toxicity, but have seen a resurgence in interest in recent years due to ever-increasing numbers of MDR pathogens (Zavascki *et al.* 2007).

Three organisms of public health importance were selected from the AMRHAI collection for the investigation into efflux-mediated tigecycline-resistance in MDR Gram-negative pathogens.

1.7.3.1 Tigecycline resistance in *Acinetobacter baumannii*

The *Acinetobacter* spp. first came to prominence in the 1970s as nosocomial pathogens, with the majority of isolates displaying susceptibility to commonly used antibiotics (Towner, 2009). The most clinically important species is *Acinetobacter baumannii* and multidrug-resistant (MDR) strains have emerged in the past two decades as nosocomial pathogens affecting severely debilitated patients and often giving rise to outbreaks (Hanlon 2005, Dijkshoorn *et al.* 2007) where it can be difficult to eradicate.

A. baumannii has a plethora of potential body sites it may colonise, with infections including bacteraemia, pneumonia, meningitis, urinary tract infections and wound infections (Maragakis & Perl, 2008) but the most clinically important is in ventilator associated pneumoniae (Livermore, 2009). Mortality from *Acinetobacter* infections has been reported as high and ranges

from around 52% (Dijkshoorn *et al.* 2007) to 75% (Smith *et al.* 2007). But as many *A. baumannii* infections are in immunocompromised/debilitated patients, little is known about this organisms' pathogenicity and mortality figures are the subject of much debate (Dijkshoorn *et al.* 2007).

A. baumannii has become notoriously resistant to antibiotics, for instance, the cell membrane is much less permeable than in *E. coli* and compared even with *P. aeruginosa*, cephalosporin permeability is 5-fold less in *A. baumannii* (Vila *et al.* 2007). When combined with the expression of several efflux pumps, these features make *A. baumannii* a particularly difficult pathogen to treat. Common treatment options for *Acinetobacter* infection used to include carbapenems imipenem and meropenem, but these have become less effective due to increasing prevalence of MDR *A. baumannii* with OXA- or metallo-carbapenemases (Livermore, 2009). Other options include aminoglycosides, which are still used in combination therapy for *Acinetobacter* infections and the β-lactamase inhibitor sulbactam which has unusual intrinsic activity against *Acinetobacter* and is also used in combination with other agents (Dijkshoorn *et al.* 2007).

However, there are even fewer therapeutic options for patients infected by the rising numbers of carbapenem-resistant strains, which are often susceptible only to polymyxins and tigecycline, but with resistance sometimes noted even against these agents (Peleg *et al.* 2007, Moffatt *et al.* 2010). *A. baumannii* is known for its pandrug resistance (PDR) potential (Falagas & Bliziotis, 2007), that is, resistant to all routinely tested antibiotics (Magiorakos *et al.* 2011) and although PDR is rarely reported, it has important public health implications.

AMRHAI has previously reported on a pair of MDR *A. baumannii* isolates belonging to the UK epidemic strain, OXA-23 clone 1 collected from a patient in intensive care before and after a week of tigecycline therapy. Tigecycline-resistance in the post-therapy isolate resulted from AdeS-mediated up-regulation of the AdeAB-TolC efflux pump (Hornsey *et al.* 2010a). This mechanism for tigecycline resistance has been described in other isolates (Ruzin *et al.* 2007). It remains unclear, however, whether the up-regulation of the AdeAB-TolC efflux pump is the only event in the acquisition of tigecycline resistance or whether it is one of a series of global changes with broader effects on resistant isolates.

While proteomics has been used before to investigate consequences of antibiotic resistance in *A. baumannii* (Fernández-Reyes *et al.* 2009; Vashist *et al.* 2010; Yun *et al.* 2008), no proteomic studies have been carried out involving tigecycline resistance.

1.7.3.2 Tigecycline resistance in *Enterobacter cloacae*

Enterobacter cloacae is an important nosocomial pathogen, particularly in neonatal and paediatric intensive care units (ICUs) where it often causes outbreaks (Dalben *et al.* 2008). It is a versatile pathogen, as infection can manifest through lower respiratory tract infections, urinary tract infections, bacteraemia, meningitis, endocarditis and skin and soft tissue infections (Sanders & Sanders 1997). Like *A. baumannii*, it causes opportunistic infections in debilitated patients and mortality from these infections is potentially high (Liu *et al.* 2004).

Treatment of *E. cloacae* infections can be problematic, owing in part to chromosomal AmpC β-lactamases, which can be induced by β-lactam exposure and confer intrinsic resistance to certain antibiotics *e.g.* cephalosporins (Paterson, 2006). In combination with decreased permeability, AmpC de-repression can confer resistance to a broader range of antibiotics including many 4th generation cephalosporins and even carbapenems (Doumith *et al.* 2009). Resistance to the latter is steadily increasing, leaving few effective therapeutic options available except polymyxins and tigecycline (Livermore *et al.* 2008).

Tigecycline has good *in vitro* activity against many *Enterobacter*iaceae (Hope *et al.* 2010), although multi-resistant *Enterobacter* spp. are among the least susceptible, often with MICs close to the current susceptible breakpoint (1mg/L) (Andrews & Howe 2011). Also, the broad substrate ranges of many efflux pumps can complicate the choice of appropriate treatment regimens. Furthermore, full resistance to tigecycline has been reported to be conferred by the RamA-mediated up-regulation of the AcrAB-TolC efflux pump (Keeney *et al.* 2007).

AMRHAI has previously reported on a tigecycline-susceptible and -resistant pair of clinical *E. cloacae* isolates from a single patient (Hornsey *et al.* 2010b). They were recovered before and after ciprofloxacin therapy, which may have selected for the up-regulated AcrAB expression that was

responsible for tigecycline resistance. While this resistance mechanism has been previously described in other isolates (Pérez *et al.* 2007) and investigations into efflux-mediated resistance in *E. cloacae* have been reported (Keeney *et al.* 2007; Pérez *et al.* 2012). No known studies have attempted to use proteomics to further probe and characterise the proteins involved in this tigecycline resistance mechanism.

1.7.3.3 Tigecycline resistance in *Serratia marcescens*

Serratia marcescens is an important opportunistic nosocomial pathogen, it is ubiquitous in the environment and has a broad host range including plants and both vertebrates and invertebrates (Van Houdt *et al.* 2007). *S. marcescens* is capable of causing infections in a broad range of sites including: bloodstream infections, conjunctivitis, pneumonia, urinary tract infections, meningitis, otitis externa and gastroenteritis (Hertle, 2005; Voelz *et al.* 2010). *S. marcescens* is also frequently associated with outbreaks, particularly in neonatal units, where this organism is a problematic and increasingly reported pathogen (Voelz *et al.* 2010). Like the two previous organisms selected for investigation of tigecycline resistance, *S. marcescens* also has a high mortality rate in debilitated patients, particularly in neonates (Maragakis *et al.* 2012).

Treating *S. marcescens* infections can be problematic due to the inherent resistance to many antibiotics, including members of the quinolones, β-lactams, macrolides, tetracyclines and polymyxins (Fritsche *et al.* 2005; Mahlen, 2011). *S. marcescens* also produces chromosomal AmpC β-lactamase which confers resistance to an even wider range of β-lactams. If these enzymes are de-repressed, the potential consequences can include resistance to carbapenems, which are frequently used as agents of last resort (David *et al.* 2006). Aside from the carbapenems, often the only remaining therapeutic option is tigecycline, which has reasonable activity against *S. marcescens,* although it is less susceptible than other *Enterobacter*iaceae (Livermore *et al.* 2008). Efflux-mediated resistance is also known to cause elevated MICs for members of the *Enterobacter*iaceae (Hornsey *et al.* 2010b; Ruzin *et al.* 2005) and since *S. marcescens* possesses multiple efflux pumps (Begic & Worobec, 2008; Hornsey *et al.* 2010c), it is capable of conferring resistance to a wide variety of unrelated compounds. The broad substrate ranges of many efflux pumps can complicate

the choice of appropriate treatment regimes and in an intrinsically resistant species such as this, efflux-mediated resistance to multiple antibiotics could make it an extremely difficult infection to clear.

AMRHAI has previously reported a *S. marcescens* clinical isolate, SM346, with resistance to tigecycline (MIC = 16 mg/L) attributed to up-regulation of the SdeXY-HasF tripartite efflux pump (Hornsey *et al.* 2010c). Comparative, quantitative proteomics techniques were employed to compare the proteome of SM346 against the *S. marcescens* type strain NCTC 10211 with the aim of characterising the proteins associated with this efflux-mediated resistance mechanism.

1.8 Detecting and interpreting antibiotic resistance

Understanding the molecular mechanisms responsible for antibiotic resistance is imperative in adapting patient treatment. It can help in the identification of new drug targets and hence lead to the discovery of novel antibiotics. Alternatively, it can provide the means to inactivate/circumvent resistance mechanisms and grant renewed activity to formerly ineffective drugs. For example, combination therapies have proved very successful in circumventing ESBL-mediated resistance with the use of β-lactamase inhibitors *e.g.* clavulanate with ampicillin or tazobactam with piperacillin (Lee *et al.* 2003). To aid this understanding of resistance mechanisms, we must know more about the proteins involved in resistance. For instance drug design and resistance mechanism determination are often based around the crystal structures of proteins *e.g.* efflux pumps and porins, antibiotic-modifying enzymes (Simmons *et al.* 2010).

Mechanisms of antibiotic resistance may be inferred from the results of classical susceptibility testing methods, such as disc diffusion and minimum inhibitory concentration (MIC) assays (Andrews & Howe, 2011). These techniques give a resistance phenotype and allow mechanisms of resistance to be inferred through 'interpretative reading' (Livermore *et al.* 2001), a strategy which analyses the susceptibility of an isolate to a range of compounds and compares the patterns of different classes to elucidate underlying mechanisms of resistance. The inferred mechanism allows healthcare professionals to advise the appropriate antibacterial regimen for

patient treatment based on the determined susceptibilities. It also allows laboratories to monitor the prevalence and spread of resistance, but it can be time consuming and remains an inexact science.

There have been recent advances in MALDI-TOF mass spectrometry which allow rapid detection (1-2 hours) of carbapenemase enzymes in bacteria (Burckhardt & Zimmermann, 2011). But the precise mechanism of resistance can still be elusive and further tests must be done to determine the resistance phenotype of the organism. For example, isolates with reduced porin expression would give a negative result for carbapenemase production, but are still resistant to the antibiotic (Wozniak *et al.* 2012).

To provide more information on these mechanisms, certain proteomics techniques may reveal more than conventional assays and could prove useful in furthering understanding of resistance mechanisms and allow more accurate interpretation of resistance phenotypes. Current molecular approaches to dissect out mechanisms of antibiotic resistance include, among others: PCR screening for resistance genes (simplex, multiplex or Real-Time PCR), DNA sequencing, microarrays and other hybridisation technologies (Woodford & Sundsfjord, 2005). However, DNA-based methods only provide information on the presence of a resistance gene and are limited by the availability of DNA sequence information. Even quantitative RT-PCR, which can generate data on the levels of mRNA transcribed from a resistance gene, does not give reliable data *e.g.* mRNA levels are not a direct reflection of translated protein content in the cell (Graves & Haystead, 2002). The study of proteins also gives the advantage of quantification, the expression levels of individual proteins may be measured in response to environmental stimulation *e.g.* addition of an antibiotic. Despite this, DNA-based techniques are still required, as mechanisms of resistance require information on both the genotype and phenotype to be fully understood. Proteomics may provide an alternative, but complementary approach to elucidate mechanisms of antibiotic resistance.

1.9 Proteomics

1.9.1 Complexity of the Proteome

The term 'Proteome' was coined by Marc Wilkins in 1994 as an analogy to the entire PROTEin complement encoded by a genOME. Proteomics encompasses the extraction, separation, analysis and eventual identification of proteins expressed in the cell/organism of interest *under a defined set of conditions* (Wasinger *et al.* 1995). The proteome of a cell is highly dynamic and complex, the expression levels of proteins will fluctuate over time as the bacterium, in this case, continually adapts to changes in its environment. By visualising and identifying components of the proteome, a vast wealth of information may be acquired not only on the effects of an environmental change (*e.g.* expression of a resistance enzyme in response to an antibiotic), but how this change affects other parts of the cell (*e.g.* effect of antibiotic on bacterial cell wall synthetic pathways) (Graham *et al.* 2011). In what is termed comparative proteomics, comparing two extracts of the same isolate under two different conditions will return two lists of proteins. The differences in these lists may be assigned to the condition that prompted their expression (*e.g.* high osmolarity), allowing the elucidation of which proteins are important or required for the condition (*e.g.* outer membrane porins) and the characterisation of the physiological response.

Furthermore, proteomics techniques enable users to study the expression levels of proteins, something which cannot be inferred from DNA sequence analysis. There are transcriptomics techniques available to study gene expression (Croucher & Thomson, 2010), but mRNA levels are known to correlate poorly with protein expression levels (Laurent *et al.* 2011). This is because mRNA transcription is only the first step in protein synthesis, proteins can have many isoforms and can be further regulated by protein turnover, secretion and truncation. Often proteins are regulated with post-translational modifications (PTMs) such as phosphorylation, acetylation, ubiquitylation, glycosylation, although the potential number of modifications could be up to several hundred (Graham *et al.* 2011). This level of complexity needs to be directly investigated, as the analysis of proteins gives the closest indication of the organisms' phenotype which cannot be reliably predicted from nucleic acid data.

1.9.2 Proteomics techniques

The modern approach to proteomics consists of two basic elements: separation of the complex mixture of proteins and identification of the individual proteins by mass spectrometry (MS). The techniques discussed in this section are summarised in Table 1.3. Separation techniques are either i) gel-based methods or ii) gel-free methods. Gel-based methods include sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE). This well-established technique uses the detergent SDS to denature and negatively charge proteins, which allows them to move in the same direction under an electric current. Running the proteins through a porous polyacrylamide gel allows separation on the basis of molecular mass and staining with *e.g.* coomassie blue, silver stain or SYPRO ruby, allows visualisation of the separated proteins. SDS-PAGE is simple to perform, is reproducible and can separate a wide range of molecular weights (10-300 kDa) (Graves & Haystead, 2002). SDS-PAGE also acts as a preliminary separation step in the proteome-wide identification of proteins. For example, one may cut out the protein profile and slice it into sections which are digested to peptides and eluted to give a fractionated mixture of peptides, allowing easier analysis by LC-MS. This method is known as GeLC bottom-up proteomics (Graham *et al.* 2007).

Isoelectric focusing (IEF) separates the proteins from complex mixtures based on their native charge by employing a ployacrylamide gel with an immobilised pH gradient (IPG), where the pH gradient is fixed into place by charged ampholytes. Under an electrical current, the proteins will migrate until they reach a pH which negates their surface charges (*e.g.* on residues like lysine and arginine) leaving the protein with a net surface charge of zero, known as the isoelectric point (pI) of a protein. IEF is an established method for the classification of beta-lactamases, comparing enzymes from clinical isolates with known beta-lactamases and reading the pI from these comparisons. While once the gold standard for ESBL identification, it has now been superseded by PCR-based methods as many ESBLs have identical pIs and are more difficult to distinguish (Sharma *et al.* 2010).

	Technique	Advantages	Disadvantages	Application	Reference
Separation	SDS-PAGE	Rapid, easy and cheap - extremely well-characterised technique	Apart from a separation profile and presence/absence of bands, not much information is gained on the sample content	To view changes in protein profile when comparing against other organisms or conditions; as an initial separation step in the MS/MS analysis of whole-cell extracts	Graves & Haystead, 2002
	2DGE	Separates proteins into single spots on a gel, in the order of hundreds to thousands - highlights differences between samples.	Single spots may contain more than one protein, time- and labour-intensive technique	High resolution separation of protein extracts, tryptic peptides can be identified on simple MALDI-TOF MS instruments	Wittman-Liebold, 2006
	LC	May separate proteins that are insoluble in gel-based analyses; increased automation, less manual handling of sample	May exclude gel-soluble proteins; more technical and expensive than *e.g.* SDS_PAGE	Increasingly used as the separation of choice to fractionate whole cell digests, particularly if gel-insolubility is an issue	Edelmann, 2011
MS analysis	MALDI-TOF	Rapid and cheap MS; relatively easy to operate	Needs a pre-separation step *e.g.* 2DGE, to identify protein digests	Recently, MALDI-TOFs have been used to speciate bacteria based on their spectral profile	Aebersold & Mann, 2003
	LC-MS/MS	High sensitivity, high resolution; able to analyse whole cell extracts without the need for a pre-separation step; can sequence the peptides for improved identification	Specialist required to operate and analyse data, machines are very expensive to purchase and maintain	Able to perform protein 'sequencing' to determine amino acid sequence of protein	Yates, 2009
Quantification	DIGE	Excellent gel-to gel reproducibility within an experiment; quantitative separation; no MS/MS needed for protein ID	Slow and labour intensive; also still requires gel-based separation so many gel-insoluble proteins will be lost	Comparative expression proteomics studies, particularly good for studying the how a change in conditions affects the expressed proteome	Madeira, 2011
	metabolic labelling	Label actually incorporated into protein via amino acids; accurate quantification	To quantify a protein, a peptide carrying the labelled amino acid must be detected	A good approach for experiments using very small protein samples	Becker & Bern, 2011
	iTRAQ/TMT	Able to multiplex up to 8 different samples in the same reaction;	An ion with a similar mass to the iTRAQ reporter ions may be mistaken for one, quantification can be affected	Investigating the effects of many changes on one organism, or comparing many organisms against each other	Pichler, 2010
	label-free	No expensive labels required, just software	Not as reproducible as label-mediated quantification	Any quantification application where labelling is not an option or is too expensive	Porteus, 2011

Table 1.3 Summary of some of the proteomics techniques mentioned in section 1.9.

Two-dimensional gel electrophoresis (2DGE) combines the two previously described techniques to increase separation of all proteins in a sample. It was successfully used by O'Farrell (O'Farrell, 1975) and works by placing a focused IPG gel on top of a larger SDS-PAGE gel, the bands of focused proteins will be again separated further by molecular weight, leaving distinct 'spots'. The aim is to separate individual proteins from the mixture, although this is not always possible as some spots may contain one or more proteins with a similar pI and molecular weight. However, these unknown spots can be excised and analysed by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) MS; the resulting spectral 'fingerprint' can be searched against online databases to identify the proteins (Aebersold & Mann 2003).

Major shortcomings of 2DGE include the poor representation of basic and membrane proteins, as well as limited dynamic range and the potential for 'hidden' proteins. 2DGE spots may be made up of more than one protein (two or more may have similar pIs and molecular weights) (Wittmann-Liebold *et al.* 2006). These disadvantages of 2DGE demonstrate the choice of method depends heavily on the type of sample and its complexity and thus sometimes, gel-based methods may not be applicable.

There are also various gel-free methods to analyse complex mixtures of proteins, a major advantage of gel-free methods is that a greater number of proteins can be detected and the sample preparation time is reduced (although the data analysis time may be greater). Liquid chromatography (LC) is a method of sample separation so that individual compounds may be identified from a mixture. This can be achieved by exploiting the hydrophobic/hydrophilic properties of the compounds to be separated. By passing these compounds through a gradient of organic solvent (referred to as the mobile phase) over a polar coating (the stationary phase) *i.e.* the more hydrophobic a molecule is, the quicker it will travel along with the gradient. There are increasing applications for this technique due to its ability to analyse large fragile molecules such as intact proteins (Zhou *et al.* 2012). The most common approach to protein identification is via bottom up proteomics, which involves digesting the proteins to peptides, separating these via LC and directly injecting into a mass spectrometer. LC-MS may be used to produce a peptide

fingerprint or LC-MS/MS (tandem mass spectrometry) is used to derive the amino acid sequence of detected peptides (Frese *et al.* 2011).

For a greater degree of peptide separation, sometimes required for biological mixtures where their complexity is too great even for highly sensitive MS equipment, one may use multidimensional-LC. This technique simply utilises two or more consecutive LC separation methods to further separate complex mixtures. It is required for the analysis of labelled protein mixtures *e.g.* iTRAQ experiments (see quantification section 1.9.4). Also, reversed-phase LC (RP-LC) and strong cation exchange LC (SCX-LC) may be employed in 2D-LC separations to increase separation and decrease sample complexity (Edelmann, 2011).

1.9.3 Mass Spectrometry

1.9.3.1 Ionisation

Mass spectrometry is a highly sensitive, high-throughput technique which is used to detect the molecular weight and even the amino acid sequence of a protein and/or peptide. In order to detect molecules and calculate their molecular mass, the sample must be ionised and in the gaseous phase. Mass spectral analysis of proteins may be either 'top-down', referring to the analysis of intact proteins; or 'bottom-up' referring to the analysis of digested peptides. The bottom-up approach is most commonly used, but with rapid advances in instruments and software, top-down proteomics is becoming more widely-used (Becker & Bern, 2011). A revolutionary development in MS for the analysis of peptides came with the advent of matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). Both of which allowed the ionisation of peptides by loss of protons in the gaseous phase (Graves & Haystead, 2002). MALDI acquires data through repeated laser shots and ion detection to give an acceptable signal-to-noise level for the rapid identification of proteins. The disadvantages are that there is low reproducibility between these laser shots and results are strongly influenced by sample preparation methods (Yates *et al.* 2009). ESI produces ions from solution by spraying droplets of mixed solvent-analyte towards the inlet of the mass spectrometer. As they are formed, the droplets rapidly evaporate (due to high temperature of capillary) to leave behind charged ions in the gaseous phase. (Yates *et al.* 2009).

1.9.3.2 Mass analysis

Ionisation is coupled to mass analysers which calculate the mass/charge ratio (m/z) of an ion and return its molecular mass. Popular mass analysers include the time of flight (TOF) type of instrument, which measures the m/z by determining the time it takes for the ion to travel the length of the flight tube (Aebersold & Mann, 2003). Combined with MALDI ionisation, the MALDI-TOF is one of the best-known and well-used mass spectrometers for protein/peptide mass analysis (Albrethsen, 2007).

Quadrupole mass analysers are a common mass analyser consisting of four parallel metal rods which generate an electric field. Ions are transmitted through this electric field by ascending m/z or they can be selectively held back allowing the quadrupole to filter ions of a certain m/z, acting as ion traps (March, 2009).

The orbitrap mass analyser consists of two electrodes, an outer barrel-like chamber and an inner spindle-like central axis. The ions are injected into the chamber and electrostatically attracted to the inner electrode but their centrifugal force stabilises them into a regular oscillation along the spindle electrode. Ions of a specific m/z will have specific frequency of oscillation (it is inversely proportional to the square root of m/z), so the oscillation frequency can be used to calculate the m/z of an ion with a high degree of accuracy with a high dynamic range (Hu *et al.* 2005). The obitrap is suited for proteomics due to its high resolution, high sensitivity and high mass accuracy, while its dynamic range and fast scan rate are also advantageous. The result is a fast, sensitive and accurate instrument, with good reproducibility and range of applications (Yates *et al.* 2009).

1.9.3.3 MS to analyse peptides

Mass spectrometry analysis *e.g.* by a MALDI-TOF, can generate a peptide mass fingerprint (PMF), a reproducible spectra (an average of many taken) of mass peaks for a given peptide. This PMF is searched against a database of many peptides (or against genomic data) and generates a match based on the peaks in the PMF (Park *et al.* 2003). However the searching assumes that the peptides come from one protein, so peptide mixtures can return poor results (Shevchenko *et al.* 1996).

Two mass analysers can be used in tandem to perform MS/MS analysis, for example quadrupole mass analysers can be used in combination *e.g.* triple-quadrupole MS, these instruments use one quadrupole to trap and analyse ions, the second for fragmentation of the analyte and the third for trapping and analysing the fragmented ions or product ions (Graves & Haystead, 2002). This analysis returns two mass spectra (hence tandem MS) and the comparison of both allows more accurate identification of the analyte. MS/MS instruments are able to ascertain the amino acid sequence of a peptide by fragmenting the molecule about its amide (or peptide) bonds via bombardment withinert gas molecules, such as nitrogen or argon (Seidler *et al.* 2010). The ion fragments (product ions) are designated a, b, c or x, y, z ions depending on whether they contain the N- or C-terminus of the original peptide (precursor ion). Because of this designation, different sequences are generated depending on which direction the molecule is fragmented *e.g.* from the N- or C-terminus, akin to the 3' or 5' direction in nucleic acid analysis. From the compilation of all possible fragments of the peptide it is possible to calculate the sequence allowing incredible accuracy when assigning protein identifications (Seidler *et al.* 2010).

1.9.4 Quantitative proteomics techniques

To add an additional layer of information to the results of proteomics identifications, various techniques may be employed to measure protein abundance. The measured abundances can be compared between experiments to determine expression changes related to virulence, adaptive responses, antibiotic resistance or any other condition under investigation. For example, this approach allows identification of proteins that are present in both isolates and expression of these proteins may be affected by the addition of an antibiotic. This could include proteins which have important roles in resistance, but are present in both sensitive and resistant organisms, such as OM porins. There are two main approaches for the quantification of protein levels, label-based methods and label-free methods, for which examples are given below.

1.9.4.1 Label-based methods

In terms of gel-based protein separation, difference in-gel electrophoresis (DIGE) which utilises fluorescent tagging of two different protein samples using two different dyes, is currently the best method of quantification and once was considered the gold standard in quantitative proteomics (Jung *et al.* 2005). The labelled protein samples are pooled together and run in the same gel, which eliminates the reproducibility issues associated with 2DGE. After image acquisition, software is utilised to create a superimposed image of the two gel profiles, to compare the two samples. This specialist software can also interpret changes in the CyDye-labelled protein spot size and intensity and give a ratio of the difference in protein expression between the two samples. DIGE does have its disadvantages, for instance sample preparation procedures must be identical, as biological variation accounts for most of the gel-to-gel variation observed (Zech, *et al.* 2011). Also many proteins (*e.g.* membrane proteins and high pI proteins) are not soluble in polyacrylamide gels. However, this technique has been used with great success (Alteri *et al.* 2009; Fernández-Reyes *et al.* 2009; Madeira *et al.* 2011) and despite its shortcomings, is still widely used today.

An important means of quantifying proteins in mixtures via LC/MS analysis has been to use stable isotope probe labelling, which allows gel-free separation and analysis of proteins. Examples of this include; isotope-coded affinity tags (ICAT), which consist of; a reactive group to label the cysteine side chain, an isotopically coded linker and a tag for the isolation of tagged peptides. Two samples are labelled with ICAT reagents (usually a heavy tag and a light tag), then mixed and digested together. Upon MS analysis, the ratio of the two labelled tags is used to relatively quantify levels of tagged peptides (Becker & Bern, 2011). A drawback of this technique is that only cysteine-containing peptides can be quantified.

Later, this technique gave rise to isobaric tags for relative and absolute quantification (iTRAQ), which uses a set of isobaric reagents to multiplex up to four, six or eight samples. Labelling of the samples with four different mass-tags generates molecules with similar or exact molecular weights and shows as one large peak in MS scans. Upon fragmentation *e.g.* with a higher-energy collisional dissociation (HCD) cell, the low-molecular weight reporter ions generated all have different masses and their ratios can be calculated to allow quantification of the

proteins they labelled (Pichler *et al.* 2010). While iTRAQ offers high sensitivity and no amino acid bias (no specific requirement for cysteine or arginine), the reporter ions generated could be iTRAQ reagents or just peptide fragments and this ambiguity only worsens with sample complexity, so quantification is not absolute, nor 100% accurate (Wu *et al.* 2009).

There is also metabolic labelling *e.g.* using radiolabelled C^{13}/H^{2}-arginine, known as stable isotope labelling with amino acids in culture (SILAC). It is a commonly used approach to quantification but is limited by the need to grow cultures in a specific media. However, Vogels and colleagues showed the advantages of SILAC in an epithelial cell model of *Salmonella typhimurium* infection, where both host and pathogen protein levels could be quantified, revealing host proteins that have a role in infection (Vogels *et al.* 2011).

1.9.4.2 Label-free methods

Label-free method of protein quantification are currently gaining momentum as they do not require complicated sample preparation steps (such as labelling) and are therefore relatively inexpensive and applicable for any sample. However, while label-free methods show a better degree of reproducibility than labelled methods, quantification is more reliable using protein labelling (Li *et al.* 2012). Label-free methods may estimate the relative or absolute protein abundance, for peptides in a mixture, they include techniques such as: sample spiking, an approach which yields relative quantification data by adding an internal standard of known concentration into the peptide mixture prior to MS/MS analysis (Porteus *et al.* 2011). Spectral counting is an increasingly utilised strategy which measures protein abundance from the spectral count data, essentially the number of peptide MS/MS spectra determined for a given protein, it also correlates well with sample spiking techniques (Porteus *et al.* 2011). The main drawback of this approach is that both data analysis and methodologies are still under development, particularly with regards to the quantification of low-abundance proteins.

1.10 Proteomics to investigate bacteria and microbial physiology

While modern MS-based proteomics is still a growing field, it has already proven to be a powerful approach for the characterisation of microbial physiology. Even now, where DNA sequencing technologies are ubiquitous, studies have shown that proteomics is still able to fill important knowledge gaps. For instance, using whole genome sequencing once took years to complete an assembled genome, now it takes merely a few hours. Despite the advances, one major caveat that has persisted since the introduction of whole genome sequencing, is that vast numbers of ORFs are poorly characterised, leaving regions of the genome annotated as functionally unknown. In a study by Kolker, roughly one third of all proteins listed in NCBI were labelled as hypothetical (Kolker *et al.* 2004). Proteomics is helping to address this continued ambiguity through studies targeting a particular protein or set of proteins labelled as 'hypothetical' and attempting to assign their functions through proteomics and bioinformatics techniques *e.g.* Zhang *et al.* assigned functions to 20 hypothetical proteins in *Desulfovibrio vulgaris* (Zhang *et al.* 2006).

For decades now, scientists have attempted to use proteomics techniques for the taxonomic classification of bacteria, from SDS-PAGE profiles (Costas *et al.* 1990) to 2DGE spot maps or reference maps (Encheva *et al.* 2006). More recently, proteomics techniques are experiencing a surge of attention in the clinical setting due to the rapid nature and ease of use of MALDI-TOF MS for the identification of bacterial pathogens, which requires very little sample preparation and can return a result in minutes (Croxatto *et al.* 2011). Other groups are looking forward to using more advanced, sensitive instruments such as LC-MS/MS to identify bacteria through peptide biomarkers (Al-Shahib *et al.* 2010; Misra *et al.* 2012).

The interactions of pathogen and host can also be probed using proteomics *e.g.* eukaryotic cells infected with bacteria could be subjected to comparative proteomics. For example, Schmidt *et al.* managed to quantify proteins of *S. aureus* recovered from infection of an epithelial cell culture (Schmidt *et al.* 2010), studies such as this could give further insights into the process of pathogenesis by highlighting which host factors are required by the pathogen and which ones will prevent infection.

Examples of recent use of proteomics techniques to aid in the understanding of bacterial pathogenicity and physiology include: Alteri & Mobley, who analysed the changes in the *E. coli* proteome from growth in human urine compared to growth in LB broth. They identified a number of proteins that were expressed only upon growth in urine, providing a host of factors which may be required for UTI pathogenicity *e.g.* six different iron scavenging proteins and an array of attachment/adhesion proteins were expressed upon growth in urine (Alteri & Mobley, 2007). Soares *et al.* used 2DGE and iTRAQ combined with LC-MS/MS to analyse the proteome of *A. baumannii* under different states of growth: exponential, and early and late stationary phase. They found that *A. baumannii* is able to tolerate high amounts of free radicals in its later growth stages and also shows tolerance to hydrogen peroxide due to increased expression of oxidative stress defence proteins. These heightened stress defence systems may aid in the tolerance of antibiotics (Soares *et al.* 2009).

Proteomics has been used to investigate bacterial responses to many changes in the environment, including antibiotics. Often proteomics has the potential to further characterise mechanisms of antibiotic resistance, through elucidation of the previously unseen proteins and protein interactions which constitute the resistance mechanism.

1.11 Proteomics to investigate antibiotic resistance

In contrast to proteomics, DNA-based techniques offer little insight into the effects of resistance gene expression on cellular processes, as the genome does not definitively indicate which proteins are expressed under the conditions being studied. This project aims to evaluate whether proteomic methodologies can be applied to enhance our understanding of antimicrobial resistance. The aim is to provide a more comprehensive overview of resistance and how it impacts on the bacterial cell. This could lead to the development of novel molecular methods to screen for resistance, resulting in better patient management and more rational use of antibiotics. It might even highlight targets for further antibacterial research.

Although a relatively new concept, using proteomics to study antibiotic resistance is an increasingly attractive approach and has been proven effective in previous studies. For example,

Zhang (Zhang *et al.* 2008) and Xu (Xu *et al.* 2006) investigated tetracycline resistance and ampicillin resistance, respectively, in *E. coli* through characterisation of membrane proteins that showed differential expression upon addition of antibiotics. Coldham (Coldham *et al.* 2006) used cell lysates of *Salmonella enterica* to study fluoroquinolone resistance and Yun (Yun *et al.* 2008) investigated the outer membrane proteins involved in *A. baumannii* tetracycline resistance.

The resistances to be investigated in this project have key public health importance (Livermore *et al.* 2008) yet to date, no work has been published on them using a proteomics-based approach.

1.12 Aims and objectives

The overall aim of this study is to characterise the proteins involved in the selected resistance mechanisms from clinically-important pathogens, using proteomics approaches such as gel electrophoresis, quantitative labelling and mass spectrometry. It is hoped that these approaches will help to elucidate novel mechanisms, or aspects of resistance, which hitherto have been difficult to define using traditional molecular methods. In doing so will provide a global overview of the processes that are affected in the cell upon exposure to antimicrobials. This may lead to new molecular tests for complex resistance mechanisms, with implications for improved patient management and rational antibiotic use.

Three key resistances will be investigated:

1) Plasmid-mediated multidrug-resistance in *E. coli*

2) Non-carbapenemase-mediated carbapenem resistance in *K. pneumoniae*

3) Efflux-mediated tigecycline resistance in *A. baumannii*, *E. cloacae* and *S. marcescens*

2. Materials and Methods

2.1 Materials and reagents

All reagents listed were from the Plus One range purchased from GE Healthcare (Buckinghamshire, UK) unless otherwise stated.

2.2 Bacteria and culture conditions

All isolates used in this work were from the collection held by ARMRL, HPA and were cultured in Lysogeny Broth (LB) media and on nutrient agar plates at 37 °C unless otherwise stated.

The three isolates of *Escherichia coli* used were strain J53 (NCTC 50165) and two transformed J53 derivatives carrying antibiotic resistance plasmids pEK204 (IncI1 plasmid; 94kb, containing $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-3}}$) and pEK499 (IncF1A/FII fusion plasmid; 117kb, containing $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, *aac6'-Ib*-cr, *mph*(A), *catB4*, *tet*(A), *dfrA7*, *aadA5* and *sul*I) (Woodford *et al.* 2009). These transformants were referred to as J204 (NCTC 13452) and J499 (NCTC 13451) respectively and their remaining resistance genes are listed in Table 2.1.

Four isolates of *Acinetobacter baumannii* were used; a pre- and post-tigecycline therapy pair of clinical isolates designated AB210 (tigecycline-susceptible, MIC 0.5 mg/L) and AB211 (tigecycline-resistant, MIC 16 mg/L); a laboratory-selected tigecycline-resistant mutant of AB210 (designated AB210-6, MIC 64 mg/L); and a tigecycline-susceptible gene-knockout mutant of AB211 (designated AB211Δ*adeB*, MIC 0.5 mg/L) (Hornsey *et al.* 2010a). The MICs of these isolates are displayed in Table 2.2.

Three isolates of *Enterobacter cloacae* were used; a pre- and post-tigecycline therapy pair of clinical isolates, designated TGC-S (tigecycline-susceptible, MIC 0.5 mg/L) and TGC-R (tigecycline-resistant, MIC 4 mg/L) and a tigecycline-susceptible gene-knockout mutant of TGC-R referred to as TGC-RΔ*acrB* (tigecycline susceptible, MIC 0.125 mg/L) (Hornsey *et al.* 2010b).

The five isolates of *Serratia marcescens* used were: a tigecycline-resistant clinical isolate, SM346 (MIC 16 mg/L): the type strain, NCTC 10211 (MIC 0.25 mg/L): a tigecycline-resistant laboratory mutant, 10211-10 (MIC 64 mg/L): and two tigecycline-susceptible gene knockout

mutants generated from 10211-10, designated 10211-10Δ*sdeY* (MIC 0.125 mg/L) and 10211-10Δ*hasF* (MIC 0.125 mg/L) (Hornsey *et al.* 2010c).

Four isolates of *Klebsiella pneumoniae* were used: a pre- and post-meropenem therapy clinical pair, 1A and 1B (ertapenem MICs of 0.125 and 16 mg/L respectively); the *K. pneumoniae* type strain ATCC 13883 and a porin deficient isolate K2 (no expression of ompK35 or ompK36; Doumith *et al.* 2009) were used as comparators for SDS-PAGE OMP analysis.

Plasmid	**pEK204**	**pEK499**
Size	94 kb	117 kb
Incompatibility group	IncI1	IncFIA-FII fusion
Number of predicted proteins	96	139
Number of resistance genes	2	10
Resistance genes present	*bla*$_{TEM-1}$ *bla*$_{CTX-M-3}$	*bla*$_{TEM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *aac6'-Ib*-cr *mph*(A) *cat*B4 *tet*(A) *dfr*A7 *aad*A5 *sul*I

Table 2.1 Features of the two multiresistance plasmids used to transform *E. coli* J53 into its resistant derivatives.

Isolate	MIC (mg/L)														
	AMP	AUG	AZT	CAR	CLAV	CLOX	CLAX	CTX	CTXC	CAZ	CAZC	CPR	CPRC	FOX	PIP
AB210	>64	>64	64	>512	>4	>100	256	>256	>32	64	>32	>64	>32	>64	>64
AB211	>64	>64	64	>512	>4	>100	256	>256	>32	64	>32	>64	>32	>64	>64
AB210-6	>64	>64	32	>512	>4	>100	64	256	>32	16	32	>64	>32	>64	>64
AB211Δ*adeB*	>64	>64	64	>512	>4	>100	256	>256	>32	128	>32	>64	>32	>64	>64

Isolate	MIC (mg/L)														
	EDTA	IME	IM	MEM	ERP	CIP	TOB	AMK	GEN	TET	MIN	TGC	SUB	COL	PTZ
AB210	>320	>16	>32	>32	>16	>8.0	>32	>64	>32	NT	2	0.5	32	≤0.5	>64
AB211	>320	>16	>32	>32	>16	>8.0	2	4	8	NT	16	16	32	≤0.5	>64
AB210-6	>320	8	8	4	>16	>8.0	>32	>64	>32	NT	16	64	16	≤0.5	>64
AB211Δ*adeB*	>320	>16	>32	>32	>16	>8.0	2	4	8	NT	8	0.5	>32	≤0.5	>64

Table 2.2 MIC values (mg/L) for the *Acinetobacter* isolates used in this research project. AMP, ampicillin; AUG, augmentin; AZT, aztreonam; CAR, carbenicillin; CLAV, clavulanic acid; CLOX, cloxacillin; CLAX, cloxacillin plus cefotaxime; CTX, cefotaxime; CTXC, cefotaxime plus clavulanic acid; CAZ, ceftazidime; CAZC, ceftazidime plus clavulanic acid; CPR, cefpirome, CPRC, cefpirome plus clavulanic acid, FOX, cefoxitin, PIP, piperacillin; EDTA, ethylenediaminetetraacetic acid; IME, imipenem plus ethylenediaminetetraacetic acid; IM, imipenem; MEM, meropenem; ERP, ertapenem; CIP, ciprofloxacin; TOB, tobramycin; AMK, amikacin; GEN, gentamicin, TET, tetracycline; MIN, minocycline; TGC, tigecycline; SUB, sulbactam; COL, colistin and PTZ, piperacillin plus tazobactam.

2.3 Susceptibility testing

E-tests (AB Biomerieux, Basingstoke, UK) were used to test antimicrobial sensitivity in accordance with the manufacturer's instructions. Briefly, bacteria grown overnight on ISO nutrient agar were suspended in ISO nutrient broth to a density equivalent to a 0.5 McFarland standard (equivalent to the absorbance of a solution of 0.05 ml of 1.175 % barium chloride and 9.95 ml of 1% sulphuric acid at 625 nm; (Andrews & Howe, 2011). This suspension was used to inoculate ISO agar plates. An E-test strip was carefully applied to the agar plate to avoid air bubbles forming under the strip and plates were incubated at 37 °C overnight. Organisms were designated resistant if their MIC exceeded the breakpoints set by BSAC (Andrews & Howe, 2011).

2.4 Phenotype Microarrays (PMs)

PM tests 1-20 were performed in accordance with manufacturer's instructions (Biolog, via Technopath, Co Tipperary, Ireland) and as described previously (Bailey *et al.* 2008; Zhou *et al.* 2003). Bacteria were grown overnight at 37 °C on BUG+B agar plates (Biolog) and used to inoculate 15 ml of inoculating fluid (IF) IF-0a. The cell density was adjusted to 42 % transmittance (T) on a Biolog turbidimeter (equivalent to an $O.D_{540nm}$ of c. 0.38) and then diluted with 75 ml of IF-0a to give a density of 85 % T (an $O.D_{540nm}$ of c. 0.08).

Plates PM1 and PM2 (measuring carbon utilisation phenotypes) were directly inoculated with 22 ml of the 85 % T suspension. Disodium succinate and ferric citrate were added to 66 ml of the 85 % T suspension to give final concentrations of 20 mM and 2 μM respectively, this solution was used to inoculate PM3-PM8 which measure nitrogen, phosphorus and sulphur utilisation and auxotrophic phenotypes (by way of nutrient supplement utilisation). 600 μl of the remaining 85 % T suspension was diluted to 120 ml with IF-10a and used to inoculate PM9-PM20 which measure sensitivity to salt and pH stress as well as antimicrobials and antimetabolites. All plates were inoculated with 100 μl cell suspension per well.

All plates were incubated at 37 °C in an Omnilog incubator (Biolog) and were repeated in duplicate. The data were recorded as RA, a unitless value of respiration activity calculated from the

reduction of the tetrazolium dye present in each test (Bochner *et al.* 2001). Plates were monitored automatically for colour changes caused by the reduced dye every 15 min for 48 h, these timepoint entries were recorded and collated into curves of RA (respiration) over time. Curves for different strains were then compared using the Omnilog-PM software (version 2.1).

2.4.1 Analysis of PM data

The data from all plates were exported to Microsoft Excel 2003 and the RA values from each individual isolate used to create a minimum cut-off value, applied to distinguish 'growth' from 'no growth'. Substrates that permitted bacterial growth in both the test and control isolates were then selected for comparison with respect to difference in RA values *e.g.* test isolate *vs.* control isolate. New minimum cut-offs were generated to differentiate between a significant difference in growth *vs.* insignificant difference in growth *i.e.* whether the compound gave either isolate a growth advantage. Substrates that did not permit growth of either isolate or failed to show a difference in growth between isolates were omitted from further analysis.

Cut-off values were calculated in a similar manner to Morales *et al.*, using averaged RA values from negative control wells of PM plates plus the standard deviation of the RA values of the control wells; an RA above this value was considered a sign of growth (Morales *et al.* 2005). All values from all substrates deemed 'significant' were zeroed and averages were taken from duplicate readings. A student's t-test was applied to the data to confirm that any differences observed were statistically significant. Any differences >2-fold or <2-fold with p values of <0.05 were considered to reflect significant changes in phenotype. The substrates associated with these changes were compiled and those that were deemed biased were removed *e.g.* if a test isolate possessed a β-lactamase, all β-lactam substrates were disregarded from analysis.

2.5.1 Protein extraction from agar plates

Bacteria were harvested from four plates of nutrient agar using plastic loops and suspended in 1 ml of standard lysis solution containing 7 M urea, 2 M thiourea, 4 % 3-[(3-

cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS – Melford, Ipswich, UK) and 40 mM dithiothreitol (DTT). The suspension was thoroughly mixed, then 300 µl of glass beads (Sigma, Gillingham, UK) were added and the cells were mechanically disrupted using a FastPrep homogeniser (MP Biomedicals, Cambridge, UK). The suspension was pulsed for 5 cycles of 60 s at a speed of 4 m/s, followed by 60 s on ice between cycles to cool the homogenate and prevent chemical modification of proteins. This crude extract was centrifuged for 30 min at 21,000 x *g* at 4 °C (Thermo) to remove glass beads and cell debris. The supernatant was removed and stored at -20 °C until required.

2.5.2 Protein extraction from liquid culture

A starter culture of 10 ml LB broth was inoculated with a single colony from NA plates and grown overnight, with the addition of an appropriate antibiotic disc to maintain pressure for resistant strains. This was used to inoculate 100 ml of LB broth 1:100, where bacteria were grown to log phase at 37 °C with shaking at 175 rpm (Orbital shaker, New Brunswick Scientific, USA) until an OD_{550nm} of 0.7-0.8 was reached. Cells were harvested by centrifugation at 10,000 x *g* for 10 min at 4 °C in a HERMLE Z36 HK centrifuge (Labortechnik, Wehingen, Germany). The cell pellet was resuspended and washed three times in 1 ml of 100 mM phosphate-buffered saline (PBS), then resuspended in 900 µl of standard lysis solution (see protein extraction from agar plates). To reduce protein degradation, 100 µl of 10x Complete Protease Inhibitor Cocktail (Roche, Burgess Hill, UK) was added to the lysis buffer mix. 300 µl of glass beads (Sigma) were added and the cells were disrupted using a FastPrep homogeniser (MP Biomedicals) using the same settings as for the agar plate extractions. Suspensions were kept on ice between cycles to cool the homogenate and prevent chemical modification of proteins. Crude extracts were centrifuged for 30 min at 21,000 x *g* at 4 °C (Thermo) to remove glass beads and cell debris. The supernatants were removed and stored at -20 °C until required.

2.6 Bradford Assay of protein concentration

To test the protein concentration of bacterial lysates, a standard curve was created using bovine serum albumin (BSA; Sigma) over eight different concentrations; 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml using the standard lysis solution (or whatever the protein samples were solubilised in) to dilute BSA to the appropriate concentration. 5 µl of each standard concentration was added in triplicate to a 96-well plate and 5 µl of each sample was added in duplicate at two different dilutions (1:10 and 1:20, or 1:1 and 1:5 for dilute samples). 250 µl of Bradford reagent (Bio-Rad, Hemel Hempstead, UK) was added to each well, the plate was left for 2 mins and the absorbance read by an ELx808 spectrophotometer (Biotek, Bedfordshire, UK) at 595 nm. The absorbance values of each extract were converted into protein concentrations using the gradient of the BSA standard curve, all duplicate values were averaged and a final concentration was recorded.

2.7 Membrane fractionation

2.7.1 Large volume method

10 ml LB broth was inoculated with a single bacterial colony and incubated at 37 °C overnight, 1 ml of this starter culture was inoculated into 1 L of LB broth and propagated overnight to log phase. Cells were collected via centrifugation in a Sorvall evolution RC large volume centrifuge (Thermo, Loughborough, UK) by spinning at 10,000 x *g* and 4 °C for 10 min. The cell pellets were resuspended in PBS, split between five tubes and washed and disrupted with the FastPrep homogeniser (MP Biomedicals) as above to yield crude protein extracts. Crude extracts were transferred to 32.4 ml Optiseal ultracentrifuge tubes (Beckman, High Wycombe, UK) and diluted with 100 mM sodium carbonate when pelleting both inner and outer membranes or with 2 % (w/v) sodium sarcosinate (sarkosyl, Fluka: as part of Sigma) when pelleting the outer membrane only. The diluted extracts were incubated at room temperature with agitation for 30 min, then transferred to a 70Ti ultracentrifuge rotor (Beckman) and centrifuged at 115 000 x *g* for 90 min at 4 °C on an optima L-100 XP ultracentrifuge (Beckman). Membrane pellets were washed three times in

sarkosyl and the final pellets were resuspended in 50-100 µl standard lysis solution and frozen at -20 °C until required.

2.7.2 Rapid membrane fractionation (ROMP method)

A rapid protocol for OMP isolation was carried out according to Carlone *et al.* (Carlone *et al.* 1986). Briefly, 10 ml LB broth was inoculated with a single bacterial colony and incubated at 37 °C overnight. Resistant isolates were cultured in the presence of appropriate antibiotic discs to maintain selection pressure. Bacteria were pelleted at 5,000 x *g* for 10 min at 4 °C and resuspended in 1 ml of cold (4 °C) 10 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES - Sigma) pH 7.4. Cells were washed by centrifugation at 15,600 x *g* for 2 min at 4 °C (Thermo) and lysed with a FastPrep homogeniser (MP Biosciences) and glass beads (Sigma) for 5 cycles of 60 s at 4 m/s. Cell debris was removed by centrifugation at 15,600 x *g* for 2 min at 4 °C, the supernatant was transferred to a fresh tube and centrifuged at 15,600 x *g* for 2 min at 4 °C to pellet cell membranes. The supernatant was discarded and the cell membrane pellet resuspended in 0.2 ml of 10 mM HEPES buffer. The cytoplasmic membranes were solubilised by the addition of 0.2 ml 2 % sarkosyl in 10 mM HEPES buffer and incubated at room temperature for 30 mins with mixing. The outer membranes were pelleted by centrifugation at 15,600 x *g* for 30 min at 4 °C, washed once in 0.5 ml 10 mM HEPES buffer and finally resuspended in 20 µl 10 mM HEPES buffer and stored at -20 °C until required.

2.8 Biotin labelling

The biotin labelling and neutravidin capture protocols were based on the methods of Smither and Peirce (Peirce *et al.* 2004; Smither *et al.* 2007). Bacteria were grown to log phase and harvested as before (section 2.5.2), then cells were washed three times in Borate Buffered Saline (BBS), containing 10 mM boric acid, 2.3 mM sodium tetraborate, 115 mM sodium chloride, pH 8.1. The cell pellet was resuspended in 1 ml BBS containing 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce, via Thermo Fisher, Northumberland, UK). Labelling took place on ice for 30 min with gentle agitation

and the reaction was quenched by addition of 1 M Tris pH 7.5 in BBS. The labelled cells were washed three times in Tris-BBS to ensure that all unbound biotin had been removed. The cells were lysed as described previously (see section 2.5.2) on a FastPrep Homogeniser (MP Biomedicals) and cell debris was removed by centrifugation (Thermo) at 1,000 x *g* for 30 min at 4 °C. Crude protein extracts were then ultracentrifuged to pellet the labelled outer membrane proteins at 115,000 x *g* for 90 min at 4 °C and on an optima L-100 XP ultracentrifuge (Beckman) and the pellet was washed in PBS three times. The final pellet was re-suspended in PBS and the amount of protein quantified using the Bradford assay.

2.8.1 Neutravidin capture

The required amount of neutravidin resin (1 ml neutravidin resin per 5 mg protein) was packed into assembled spin columns (Pierce, Northumberland, UK) and centrifuged at 1,000 x *g* for 1 min to remove the liquid from the resin (2 ml neutravidin solution = 1 ml settled resin). The resin was washed three times in buffer A (25 mM Tris-HCl, 0.15 M NaCl, 0.5 % NP-40, 0.5 % sodium deoxycholate, 0.05 % SDS, pH 7.5) discarding the wash each time. The resin was then re-pelleted as above, the spin column capped and the labelled OMP lysates fraction was added (5 mg protein per ml resin). The resin was incubated for 1 h at room temperature with gentle mixing and then removed by centrifugation at 1,000 x *g* for 1 min. The resin was washed twice with buffer B1 (25 mM Tris-HCl, 0.65 M NaCl, 0.1 % NP-40, pH 7.5) and once in buffer B2 (25 mM Tris-HCl, 1.15 M NaCl, 0.1 % NP-40) to remove any non-specifically bound proteins. To elute the labelled proteins, the resin was incubated in 50 mM DTT in PBS for 10 min to reduce the disulfide linker and sever the link to the biotin molecule. The resin was centrifuged at 1000 x *g* for 2 min at 4 °C and the eluant was transferred to a clean tube. This step was repeated to ensure all biotin-tagged protein was removed; the eluant was then quantified and stored at -20 °C.

2.9 Sample clean-up

2.9.1 GE Healthcare clean-up kit

300 µl of the 'precipitant' reagent was added to 100 µg protein, which was vortexed and incubated for 15 min (all incubations in this procedure are carried out on ice). 300 µl of the 'co-precipitant' reagent was added and vortexed, then the proteins were pelleted by centrifugation (Thermo) at maximum speed for 5 min. The supernatant was decanted and the pellet briefly centrifuged to remove all trace of liquid. Then, 40 µl of the 'co-precipitant' reagent was overlayed onto the pellet and left for 5 min. The tube was then centrifuged for 5 min, the supernatant discarded and the pellet was dispersed with 25 µl distilled water and vortexing. 1 ml of the 'wash buffer' solution (pre-chilled to -20 °C) and 5 µl of the 'wash additive' solution were added and the pellet was vortexed until completely dispersed. Protein samples were incubated at -20 °C for at least 30 min with intermittent vortexing (roughly every 10 min), then centrifuged for 5 min. The supernatant was discarded and the pellet air-dried (for 1 min) before being re-solubilised in standard lysis solution. Preparations were either used immediately or stored at -20 °C.

2.9.2 Acetone precipitation

Proteins were transferred to a clean tube and precipitated with five times the sample volume of ice cold acetone (pre-chilled at -20 °C). The tubes were mixed and left at -20 °C for 1 h with intermittent mixing every 20 min. The protein precipitate was then pelleted in a microcentrifuge at 21,000 x *g* for 10 min at 4 °C and was resuspended in standard lysis solution. Protein concentrations were calculated using the Bradford assay (see section 2.6) before the samples were stored at -20 °C.

2.10 DIGE labelling

Proteins prepared for DIGE analysis were extracted in a modified standard lysis solution that included 30 mM Tris to raise the pH to 8.5, the optimal pH required for DIGE-labelling with

CyDye minimal dyes (GE Healthcare). The pH of protein extracts was checked with pH indicator strips (Sigma) and 50 mM hydrochloric acid and 50 mM sodium hydroxide to adjust extracts to pH 8.5. 50 µg of each protein extract used in the experiment was labelled with 400 pmol of CyDye minimal Dye Cy3 or Cy5, along with 50 µg of an internal standard labelled with Cy2 (containing equal amounts of each protein extract used in the DIGE experiment; see Figure 2.1). The CyDyes were randomised when labelling an experiment consisting of biological replicates *e.g.* three replicate extracts of each bacterial isolate were labelled alternately with Cy5/Cy3/Cy5 or Cy3/Cy5/Cy3 to check for preferential labelling. Random labelling of all samples ensures no bias towards any one dye. All incubation steps involving the CyDye compounds were carried out in a sealed polystyrene tub, away from light as they are highly photosensitive. Proteins were incubated with CyDyes for 30 min on ice and the reaction was then quenched by addition of 1 µl of 10 mM lysine per 400 pmol of CyDye and incubation for 10 min on ice. Extracts were pooled together and adjusted to 150 µl with DIGE rehydration solution (7 M urea, 2 M thiourea, 2 % CHAPS, 20 mM DTT and 0.5 % immobilised pH gradient (IPG) buffer), then immediately analysed by IEF.

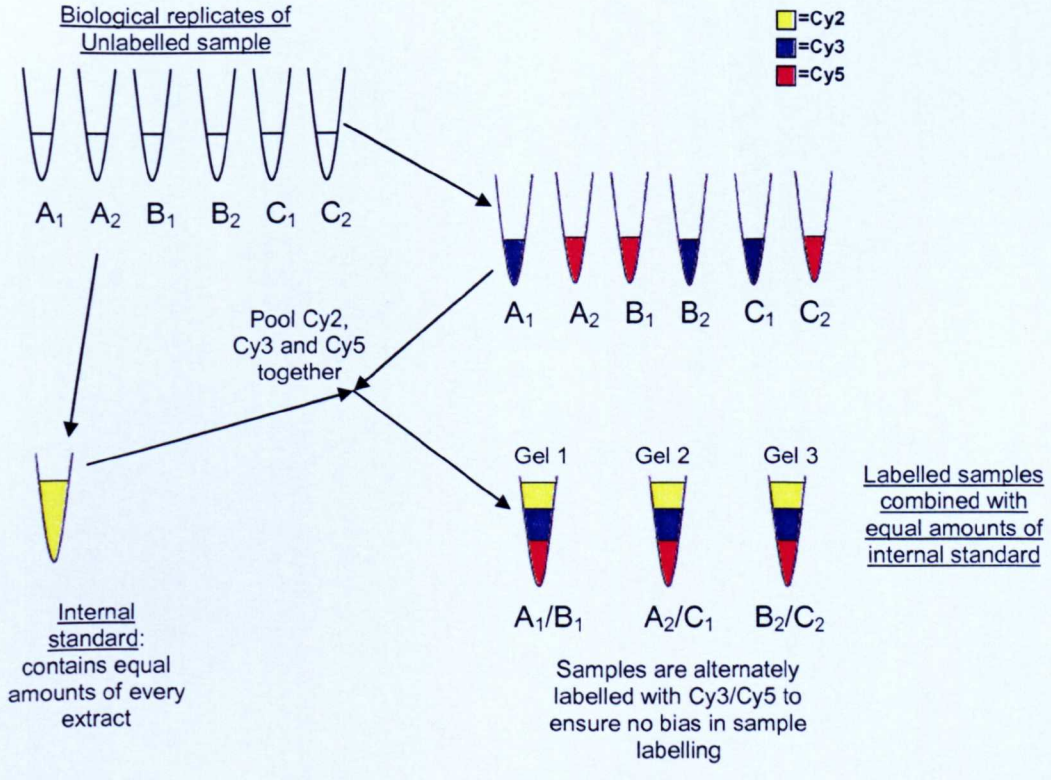

Figure 2.1 Overview of the DIGE protein-labelling process.

2.11 SDS-PAGE

2.11.1 Lab-cast gels

The OMP fractions of each isolate were boiled for 10 min in 2x sample buffer containing 125 mM Tris-HCl (pH 6.8), 20 % (v/v) glycerol, 4 % (w/v) SDS and 10 % β-mercaptoethanol (v/v). The OMPs were separated in gels containing 12.5 % acrylamide, 0.35 % bisacrylamide and 0.1 % SDS. Gels were run at 60mA for 1 h in SDS running buffer (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS), stained with Coomassie Brilliant blue (Sigma) in a 45 % methanol/10 % acetic acid mixture overnight and destained by washing with the same solution for at least 1 h.

2.11.2 Nu-PAGE gels

Nu-PAGE gels (Invitrogen, Paisley, UK) were run according to manufacturer's' instructions; 5 µg of each protein extract was mixed with 1 µl of 10 × reducing solution (500 mM DTT) and 2.5 µl 4x sample buffer (including glycerol and lithium dodecyl sulphate at pH 8.4). This mixture was made up to 10 µl with distilled H_2O and heated at 70 °C for 10 min. Ten µl of Novex sharp unstained protein standard (Invitrogen) was used as a marker and gels were run in Nu-PAGE MES SDS running buffer with 500 µl antioxidant added to the upper chamber at 200 V for 50 min. Gels were fixed in 40 % methanol for 1 h and stained overnight in either SYPRO ruby stain (Invitrogen) or Colloidal G Brilliant Blue Stain (Sigma) in 25 % methanol. Gels were destained for 1 h in 10 % methanol before being scanned in an Ettan DIGE imager (GE Healthcare) for fluorescent gels or in a ProPic II scanner (Digilab, Huntingdon, UK) for colloidal gels.

2.12 2-Dimensional gel electrophoresis

2.12.1 1st Dimension: IEF on pH 4-7 strips

Immobilised pH gradient (IPG) strips of pH 4-7 were re-hydrated overnight in a Drystrip re-swelling tray (GE Healthcare) with 340 µl re-hydration solution containing 7M urea, 2M thiourea, 2 % CHAPS, 20mM DTT, 2 % IPG buffer (pH 4-7) and 0.002 % bromophenol blue. Strips were

overlayed with mineral oil to prevent dehydration and urea crystallisation. 100 µg of protein extract was diluted with rehydration solution to a final volume of 150 µl and added to the IPG strip via cup-loading at the anode. Paper wicks were dampened with 150 µl of deionised water to carry current from the electrodes to the strips. Samples were focused using a universal focusing program (Table 2.3) and all focusing was carried out using a current of 50 µA per strip. After focusing, the second dimension was run immediately or the strips were stored at -80 °C until required.

	Voltage application	Voltage	Time
1	Step and hold	300V	3 h
2	Gradient	1000V	6 h
3	Gradient	8000V	3 h
4	Step and hold	8000V	3 h
5	Step and hold	8000V	5 h

Table 2.3 Universal IEF program used for all strips of pH 4-7 and 3-10.

2.12.2 1st Dimension; IEF on pH 6-11 strips

IPG strips of pH 6-11 were rehydrated overnight in a Drystrip re-swelling tray (GE Healthcare) with 340 µl re-hydration solution containing 7M urea, 2M thiourea, 2 % CHAPS, 2 % IPG buffer (pH 4-7) and 0.002 % bromophenol blue. To prevent protein streaking at the cathode, 12 µl DeStreak solution (GE Healthcare) was added per ml of rehydration solution. 100 µg of protein extract was diluted with rehydration solution containing 7M urea, 2M thiourea, 2 % CHAPS, 10mM DTT, 0.5 % IPG buffer (pH 6-11) and 0.002 % bromophenol blue to a final volume of 150 µl and added to the IPG strip via cup-loading at the anode. Anodic paper wicks were dampened with deionised water while cathodic wicks were dampened with water containing 12 µl DeStreak solution per ml. Samples were focused using a program designed for pH 6-11 strips (table 2.4) after focusing, the second dimension was run immediately or the strips were stored at -80 °C until required.

	Voltage application	Voltage	Time
1	Step and hold	300V	2 h
2	Gradient	1000V	8 h
3	Gradient	8000V	3 h
4	Step and hold	8000V	2 h

Table 2.4 Universal IEF program used for all strips of pH 6-11

2.12.3 Gel Casting

The polyacrylamide gels used for the second dimension of 2D GE contained 12 % acrylamide. To make six gels, 187 ml LC-grade water was added to 140 ml 40 % acrylamide:bis-acrylamide 19:1 (Bio-Rad) and 112.5 ml 1.5 M Tris-HCl pH 8.8 and stirred. To this mixture was added 4.5 ml of 10 % SDS solution (Gibco), with 5 ml 10 % ammonium persulfate (APS) and 125 µl of N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) to catalyse polymerisation. The gels were cast using an Ettan DALT 6 casting box (GE Healthcare) and overlayed with 80 % isopropanol. After 4 h of polymerisation, the gels were covered with gel storage solution (0.1 % SDS, 375mM Tris-HCl pH 8.8) and left overnight to continue to polymerise. Gels were either used immediately the next morning or kept in short-term storage (1-4 days) at 4 °C wrapped in wet paper towels.

2.12.4 IPG strip Equilibration

If the strips were frozen, they were allowed to equilibrate to room temperature for roughly 45 min. Each focused strip was then incubated in 4 ml equilibration solution containing 6 M urea, 75mM Tris-HCl pH 8.8, 30 % (v/v) glycerol, 2 % (v/v) SDS, 0.002 % bromophenol blue and 1 % DTT at room temperature for 20 min to reduce the side chains of cysteine amino acids. Strips were then incubated in 4 ml of the same solution for 20 min with 2.5 % iodoacetamide replacing the DTT to alkylate or 'cap' the reduced cysteine side chains. This reduction and alkylation prevented re-oxidation of the reduced proteins during electrophoresis.

2.12.5 2nd Dimension

Equilibrated IPG strips were placed on top of 12 % polyacrylamide gels and overlayed with 1.5 ml agarose sealing solution containing 1x TGS running buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS at pH 8.8; Bio-rad), 0.002 % bromophenol blue and 0.5 % low melting-point agarose (GE Healthcare). A molecular weight marker was prepared by adding 25 µl of novex sharp unstained protein standard (Invitrogen) to 25 µl of agarose-sealing solution, mixing and pipetting into a well moulded into the agarose layer. Gels were immersed in 1 × TGS running buffer in the lower buffer chamber while the upper buffer chamber contained 2 × TGS running buffer. Gels were run overnight at 80 V, 10 mA per gel and 1 W per gel until the dye front had reached the bottom of the gel (about 22 h).

2.12.6 Protein staining

After proteins had been separated, gels were fixed in 40 % methanol for 1 h and then stained overnight with either Brilliant blue colloidal G stain (Sigma) in 25 % methanol or in the dark with SYPRO ruby fluorescent stain (Invitrogen). The next day gels were rinsed twice with distilled water and de-stained with 10 % methanol for 1 h before imaging.

2.13 Gel imaging

For CyDye labelled gels, once the second dimension was completed they were imaged immediately without the need for fixing or staining. Gels were scanned on an Ettan DIGE scanner (GE Healthcare) with all three CyDyes scanned simultaneously at a resolution of 100 pixels. Colour images of DIGE gels were collated using ImageQuant software tools (GE Healthcare). SYPRO-stained gels were scanned with the Ettan DIGE imager after destaining at a resolution of 100 pixels and colloidal-stained gels were scanned with a ProPic II spot-picker (Digilab, Huntingdon, UK). Image exposure was adjusted to ensure that there was no signal saturation from any stained spots (resulting in unreliable gel analysis downstream). Using ImageQuant tools (GE

Healthcare) software, noise and artefacts on the gel surface were removed from the image and files were converted from .gel to .TIFF images. After scanning, gels were stored in 25 % ammonium sulphate at 4 °C until required for spot-picking.

2.14 SameSpots software for 2DGE analysis

Once all the extracts had been tested, they were labelled with the CyDyes and separated using the 2DGE protocol on gradients of pH 4-7. The .GEL image files of the separated proteins were transferred into Progenesis SameSpots software (version 3.3) where the gels were checked for image saturation (image saturation occurs when the background is measured as higher than it actually is, due to *e.g.* image exposure too high. This results in loss of information on spot area and intensity, leading to inaccurate analysis) and dynamic range (the range of potential pixels actually used in the gel image: a low dynamic range means less precision in the representation of spot intensity, therefore less precise quantification). One of the gels was selected to be a reference image, based on the resolution and quality of the gels, which gave the best representation of all gels in the experiment, on which, the spot positions would be mapped out on for the rest of the workflow. SameSpots then assigned spot positions and numbers according to what it believed to be protein spots. But, there was an opportunity to manually check assignment of spot numbers and positions, to ensure that as many as possible non-protein spots (artefacts) were disregarded. This included cropping areas that were not of interest (sides and bottom of gel) and removing very low-volume spots (caused by *e.g.* dust). When all protein spots were mapped, the proteins 'of interest' were taken forward for picking and identification. Proteins were considered to be 'of interest' if they displayed a difference in expression between isolates of 2-fold or greater and also had $p < 0.05$ (using a one-way ANOVA test) of displaying a false positive expression difference.

Spots that also appeared novel to one isolate *i.e.* a protein that was present in one isolate but not another, were also selected as 'unique' spots of interest. These were tentatively selected however, as the absence of a protein spot may not mean that it wasn't expressed, it may be present at a low level, it may have undergone post-translational modifications or an isoform with a different pI may have been expressed instead. Without other data *e.g.* a sequenced genome to

confirm the presence of a gene and the 'uniqueness' of these spots, no firm conclusions may be drawn from them.

Once the proteins of interest were selected, a protein "pick list" was generated from SameSpots software, consisting of an image of the DIGE reference gel annotated with spot numbers and areas, with a table detailing expression levels, p values and expression ratios between the two samples. This list of proteins was manually entered into the ProPic spot picking robot and overlaid onto an image of the picking gel. The proteins of interest were automatically picked and deposited into a 96-well plate, after which the picking gel was overlayed onto a 1:1 paper image of the gel to visually check all spots had been picked that were selected. The gel was re-imaged to ensure that all the right proteins had all been picked. A note was made of the pick numbers and how they corresponded to the positions in the 96-well plate. Although it would have been preferable to electronically transfer the report list into Propic rather than have the operator manually select spots, it was not possible at the time due to compatibility issues between the software packages used. Once picked, all proteins of interest were then digested as in methods section 2.16 and the peptides were submitted for identification by LC-MS/MS.

2.15 Spot picking

Gels were scanned into the ProPic 2 (Digilab) spot-picker and pick-lists were compiled by manually selecting proteins of interest for picking according to the picking list generated by SameSpots. Distilled water was used to draw in, expel and store the excised spots in a 96-well plate. Protein spots were excised at a diameter of 1.2 mm, placed into a 96-well plate and either frozen at -80 °C or immediately digested.

2.16 Protein digestion

Excised gel plugs were washed three times and incubated for 20 min in 100 µl of 50 % methanol in 50mM ammonium bicarbonate (AmBic - Fluka), then dehydrated with 100 µl acetonitrile (Fisher Scientific). Ten µl of 10mM DTT was added to each gel plug and the plate was incubated at 60 °C

for 30 min. The gel plugs were again dehydrated with acetonitrile and alkylated with 10 µl 50mM iodoacetamide in the dark at room temperature for 45 min. Once alkylated, the plugs were washed three times with 25 mM AmBic solution, dehydrated with acetonitrile and incubated with 20 µl of 20 ng/ml modified sequencing-grade trypsin (Promega, Southampton, UK) overnight at 37 °C. The next day the plate was centrifuged at 4000 x *g* for 10 min at 4 °C on a Haraeus Megafuge II plate centrifuge (Thermo) and the peptides were extracted with 20 µl of 0.1 % tri-fluoroacetic acid (TFA – Fluka) at room temperature for 1 h with shaking at 300 rpm on a Thermomixer (Eppendorf, Cambridge, UK). The plate was centrifuged as above and peptides were transferred to a clean plate and stored at -80 °C until required.

2.17 Zip-tip concentration and clean-up of peptides

Peptide extracts were de-salted and concentrated using C18 Zip-tips (Millipore, Watford, UK). The C18 column was activated by the aspiration of 10 µl acetonitrile with the retention of enough liquid to cover the column throughout the procedure, thus preventing air bubbles entering the column. The tip was washed once with 10 µl of 50 % acetonitrile and 0.1 % TFA, then washed three times with 10 µl 0.1 % TFA. Ten µl of peptide extract was bound to the column by drawing up and expelling the solution 10 times. The bound peptides were washed three times with 10 µl 0.1 % TFA, then eluted and concentrated by drawing up 4 µl of 50 % acetonitrile/0.1 % TFA and expelling the entire contents into a clean tube. Clean peptides were either stored at -80 °C until used, or spotted directly onto a MALDI target plate (see section 2.20).

2.18 MALDI target plate cleaning

The MALDI target plate (Waters, Elstree, UK) was submerged in 100 % methanol for 5 mins after which it was scrubbed with Decon 90 detergent (Decon laboratories, Hove, UK) and rinsed with distilled water. The plate was then re-submerged in methanol and sonicated in a water bath sonicator for 15 mins. The plate was then rinsed with acetone and air-dried both after sonication and again just before use.

2.19 MALDI-TOF MS plating and analysis

For peptide mass fingerprinting (PMF), 0.75 µl of clean peptides were spotted onto a stainless steel MALDI target plate (Waters) then 0.75 µl of matrix solution (10 µl of 0.1 % TFA, 495 µl acetonitrile and 495 µl ethanol containing 10 mg/ml of α-cyano-4-hydroxycinnamic acid (Sigma) was added and mixed, then left to dry. In addition to peptide extracts, 1 pmol Renin (Sigma) peptides was used as the lock mass to correct the mass accuracy with a tolerance of 0.5 Da and 1 pmol alcohol dehydrogenase (ADH - Sigma) digest was used as the calibrant to optimise the pulsed voltage settings. The ADH peptides were obtained by dissolving 1 mg ADH in 300 µl of 50 mM AmBic, then dissolving 1 mg of porcine trypsin in 500 µl of 50 mM AmBic. Five µl of the trypsin solution was added to the ADH solution and mixed, then incubated at 37 °C for 90 min. Ten µl of this digest was mixed with 990 µl of 0.1 % TFA to give c. 1 pmol ADH peptides per µl. The plate was loaded into a MALDI-TOF reflectron Mass Spectrometer (Waters, Hertfordshire, UK) operated by the Masslynx software, equipped with a 337 nm nitrogen laser and set to positive ion mode. The following voltages were applied; a source voltage of 15,000 V, a pulse voltage of 2700 V, a reflectron voltage of 2000 V and the detector (micro channel plate detector or MCP) had a variable voltage in the region of 1800-2000 V. Spectra were collected 40 times from each sample well and 20 times from each lock mass well at a rate of 2 wells per ms and a laser firing rate of 20 Hz. Ions were detected over a mass range of 800 to 4000 Da.

2.20 LC-MS/MS analysis of peptide samples (GeLC)

Peptides were analysed using online nano liquid chromatography and tandem mass spectrometry (nano LC-MS/MS) on an Ultimate 3000 Dionex nano/capillary HPLC system (Dionex) coupled to a LTQ Orbitrap mass spectrometer (Thermo Fisher, Hemel Hempstead, UK). The separations were performed on a nano analytical C18 column (75 μm id × 15 cm, 3 μm) (Dionex) using a 45-min linear gradient of 5 to 45 % solvent B (90 % acetonitrile/0.1 % formic acid) versus solvent A (2 % acetonitrile/0.1 % formic acid), then to 90 % B for an additional 5 min. MS/MS data was acquired

in a 'data-dependent' mode to automatically switch between MS and MS/MS acquisition using Thermo Finnigan Xcalibur software (version 2.0.6). The precursor ion scan MS spectra (*m/z* 440-2000) were acquired in the Orbitrap, followed by MS/MS scans in which the six most abundant peptide precursor ions detected in the preceding survey scan were dynamically selected and sequentially isolated for further fragmentation in the linear ion trap using collision-induced dissociation (CID) to generate MS/MS spectra.

2.21 Database searching

For protein identifications, generated MS/MS spectra were searched using MASCOT (Matrix science, www.matrixscience.com) against a genus-specific database curated in-house containing all non-redundant (nr) protein sequences of the target organism available on NCBI (http://www.ncbi.nlm.nih.gov/). The following search parameters were applied in MASCOT: two maximum missed trypsin cleavages; variable methionine oxidation; fixed cysteine carbamidomethylation, a state charge of up to +2, a fragment ion mass tolerance of 0.1 Da and a parent ion mass tolerance of 10 ppm. Large batch searches were carried out using Mascot Daemon and the .DAT result files were collated and viewed in Scaffold (version 3.3.2; proteome software inc., Portland, USA). Scaffold was used to validate MS/MS based peptide and protein identifications and assign probability scores using the built-in Protein Prophet and Peptide Prophet algorithms. Peptide identifications were accepted if they established minimum peptide probability of 95 %. Protein identifications were accepted if they contained at least two identified peptides and established minimum protein probability of 99 %. To further characterise hypothetical or unmatched proteins, the BLASTp algorithm (http://blast.ncbi.nlm.nih.gov/) was used to search for homologous proteins (NCBI default settings were used unless otherwise stated). The Uniprot database (www.uniprot.org) was used for information regarding the function of proteins and the web-based tools InterProScan and PSORTb (www.ebi.ac.uk/Tools/pfa/iprscan/ and www.psort.org/psortb/) were also utilised respectively to detect conserved domains and predict subcellular localisation of unknown proteins of interest.

2.22 Peptide analysis using Scaffold software

Scaffold (version 3.3.2) uses the Protein Prophet and Peptide Prophet algorithms which are run and compared with the results from Mascot Daemon. A result which satisfies both algorithms generates protein identifications with higher scores and lower false discovery rates (FDR). The FDR describes the expected proportion of incorrectly-rejected null hypotheses (or false discoveries) with regards to assigning protein identifications. Adding Scaffold into the proteomics workflow therefore gives higher confidence in protein identifications which are more robust than either program alone. Scaffold can also base a protein identification on the number of peptides detected for that particular protein, this is another measure to give better confidence in the false discovery rate *e.g.* identification of 20 peptides across a protein sequence suggests a greater likelihood for the presence of that protein, than using 1 peptide across a sequence. Protein and peptide identifications were accepted if they could be established at greater than 99.0% and 95.0% probability respectively and contained at least 2 identified peptides. Proteins that did not meet these requirements were not pursued for further analysis.

3. Results
Multidrug resistance plasmids in *Escherichia coli*

3.1 Introduction of isolates

The isolates used in this study included *E. coli* J53, a common strain used for transconjugation experiments due to its F+ phenotype and azide resistance allowing for selection of transformants (Yi *et al.* 2012). J53 was used to receive two plasmids: pEK204 and pEK499, to generate transformants J204 and J499 respectively. The resistance genes harboured by these plasmids are tabulated in methods section 2.2, Table 2.1.

To characterise the changes to the proteome of J53 caused by plasmid acquisition, the isolates were compared by 2DGE. Then their SDS-PAGE profiles were characterised by LC-MS/MS analysis to determine the effect of plasmid acquisition on the proteome. The isolates were also compared using a Phenotype Microarray (PM) system which evaluated their growth on c. 2000 substrates. The aim was to determine which compounds gave specific advantages or disadvantages to the plasmid host, J53 *i.e.* whether the plasmid provided its host with the means to grow on a substrate/metabolite, to which it had no resistance genes against.

This investigation aims to elucidate the effect of acquisition of a multi-resistance plasmid on protein expression levels and cellular processes of the host cell. There is much speculation as to why certain plasmids seem to give an advantage to their host compared with others and why those that offer no obvious advantage are retained. Proteomics provides a good opportunity to better investigate the changes in cell physiology resulting from plasmid acquisition. It is hoped proteomics could aid in the identification of promoters or cellular cofactors associated with the resistance plasmids and may provide new targets to prevent their transfer, or with the specific CTX-M enzymes, in this case CTX-M15 and CTX-M3. The worldwide spread of CTX-M is a paradigm of antibiotic resistance dissemination and any insights on how to halt the spread could be utilised against other resistance enzymes poised to flourish in a similar manner, such as the NDM carbapenemases (Kumarasamy *et al.* 2010).

3.2 2DGE separation of *E. coli* protein extracts

3.2.1 Separation over a pH 4-7 gradient

The *E. coli* transformants were the first isolates to have their whole-cell protein extracts separated by 2DGE. Initially, the extracts were separated over pH 4-7 gradients, as many of the cytosolic proteins are resolved in this pH range. The method of protein loading into the IPG strips was optimised for efficient protein delivery into the strip and subsequent gel resolution.

There are two commonly used methods, cup-loading and in-gel rehydration. The gels displayed below (Figs 3.1-3.3) were produced with cup-loading and gave better separation and resolution compared to the rehydrated gels.

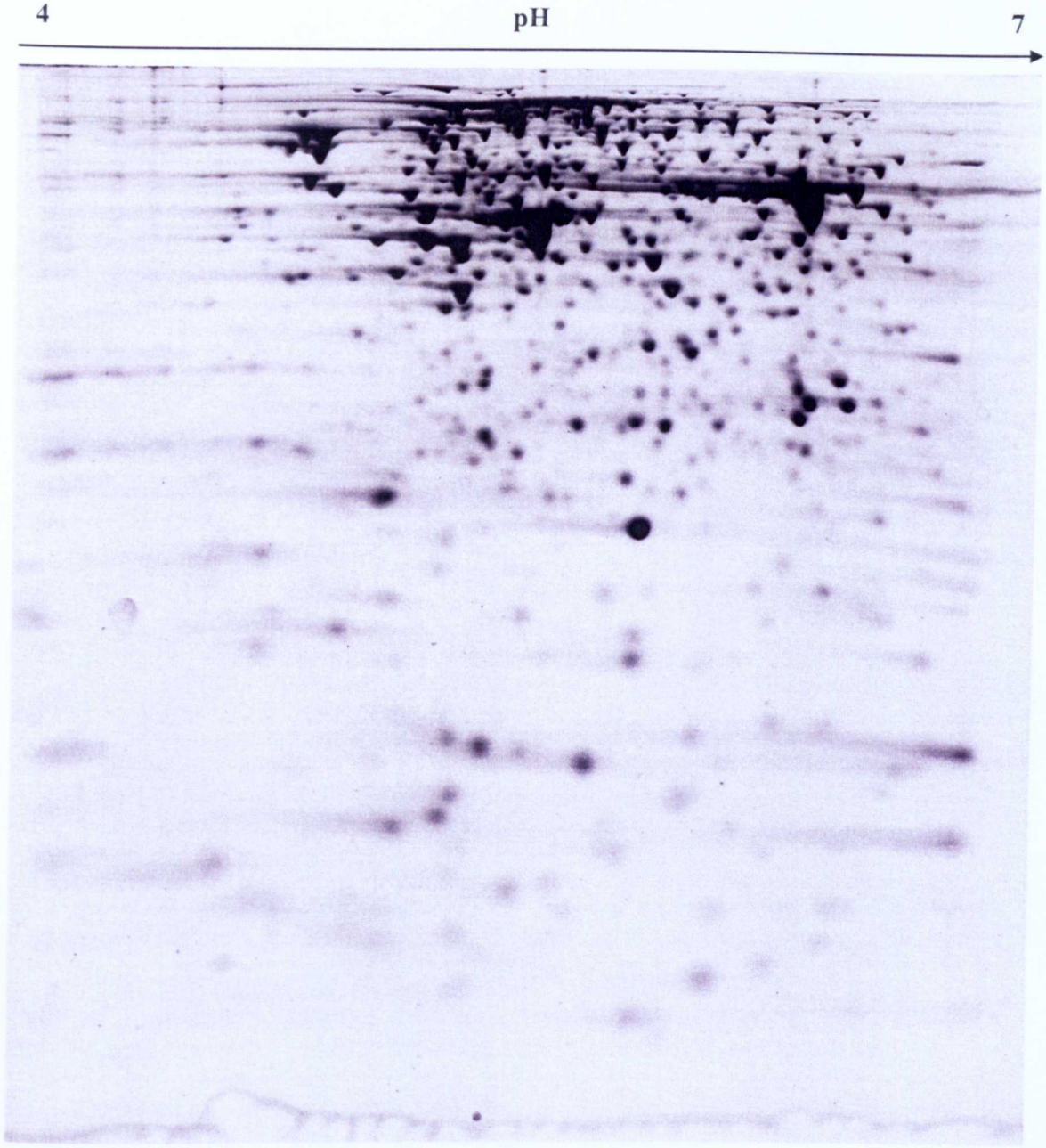

Figure 3.1 2DGE protein profile of *E. coli* J53 separated over a pH 4-7 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

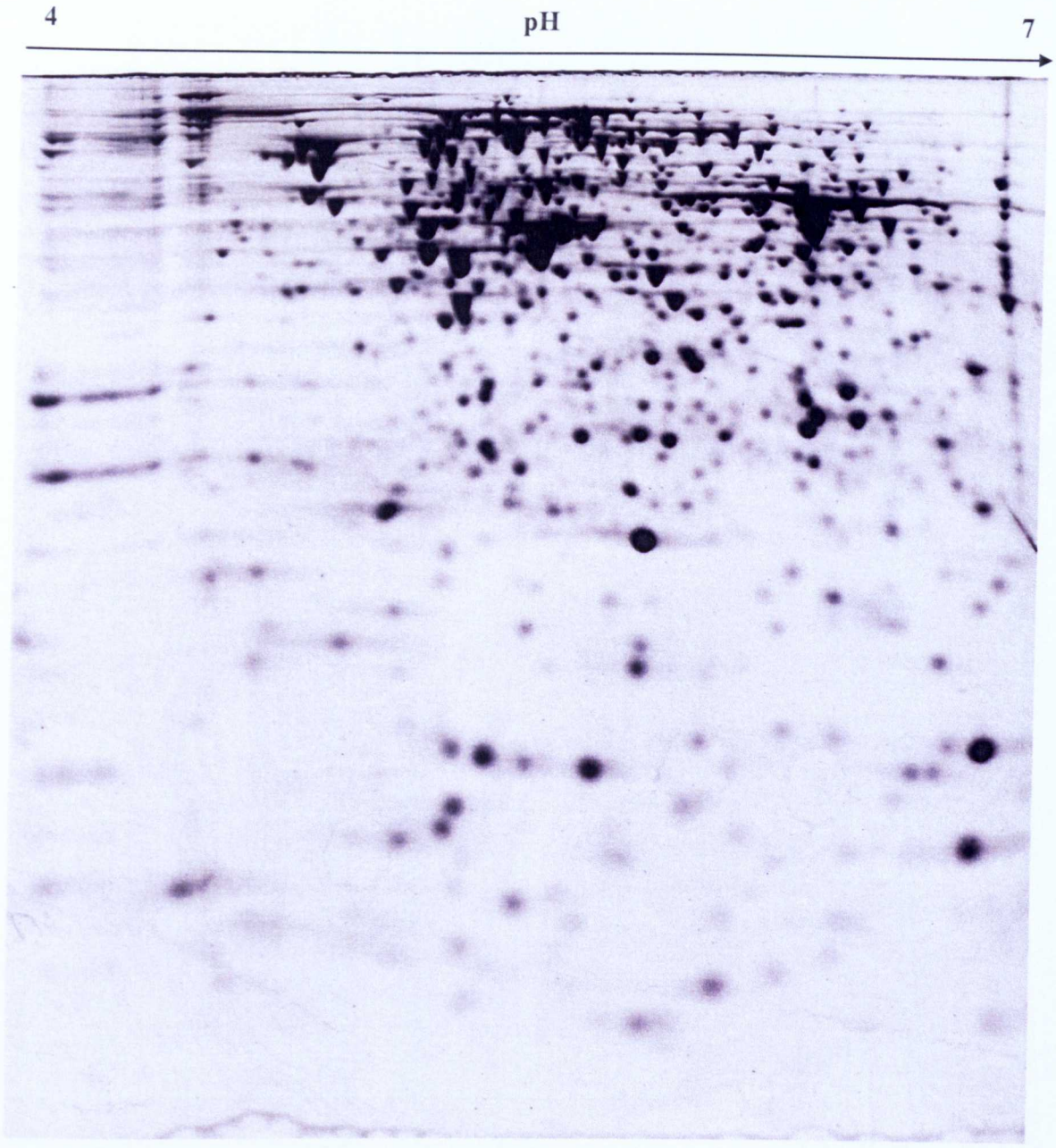

Figure 3.2 2DGE protein profile of *E. coli* J204 separated over a pH 4-7 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

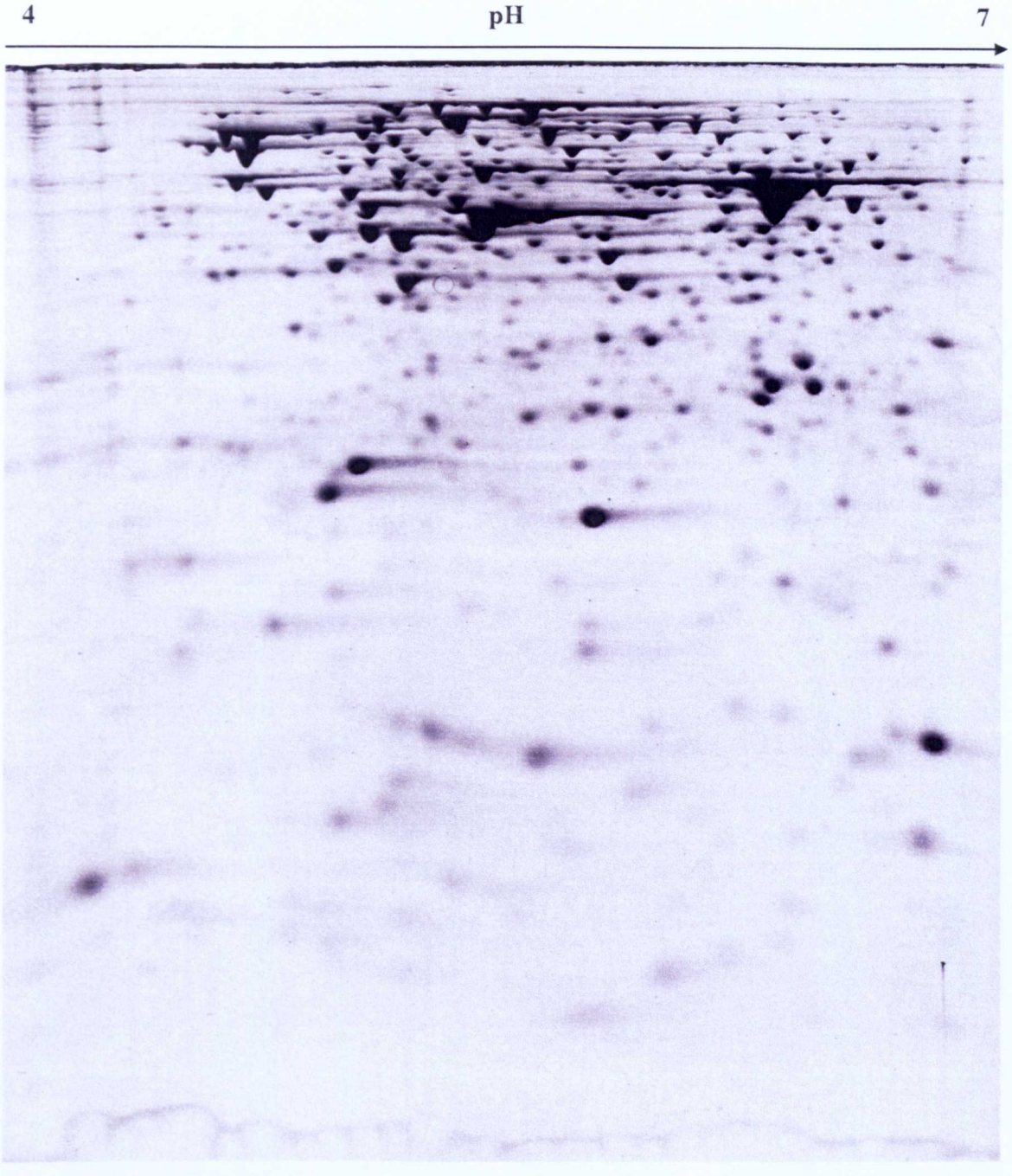

Figure 3.3 2DGE protein profile of *E. coli* J499 separated over a pH 4-7 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

3.2.2 Separation over a gradient of pH 6-11

One of the original aims of this study was to visualise the CTX-M ESBLs via 2DGE, however, as the pI of these proteins is around pH 8-9 and therefore would not separate on pH 4-7 gradients. Therefore, to better visualise these proteins on the gels, the same extracts were separated by pH 6-11 gradients and compared using Proteomweaver software (version 3.0).

While running two separate pH gradients (of pH 4-7 and 6-11) increases the time and labour compared to running a pH 3-10 gradient, the resolution of proteins will be much greater. This would yield a higher number of separated spots and allow a more thorough characterisation of the proteomic response to plasmid acquisition.

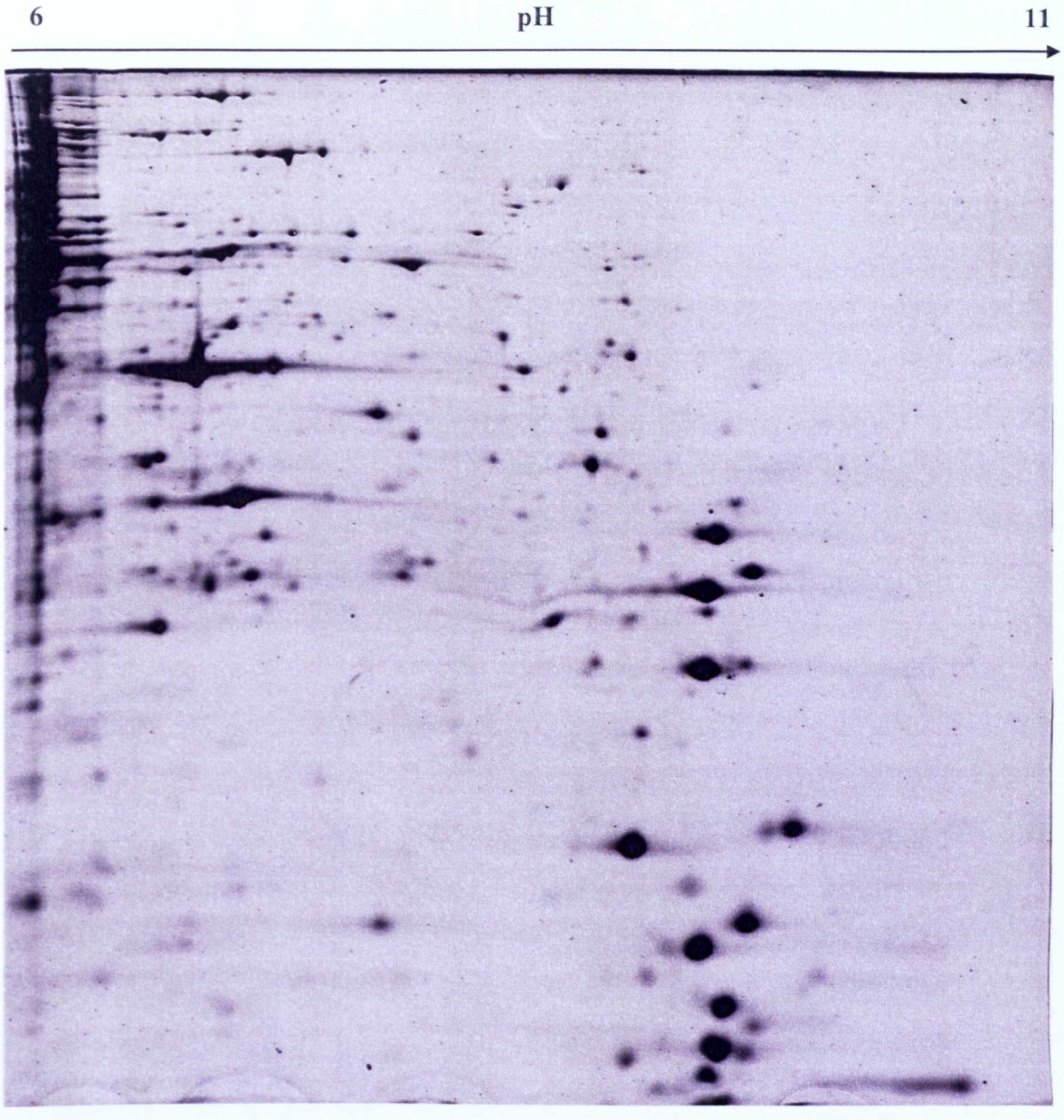

Figure 3.4 2DGE protein profile of J53 separated over a pH 6-11 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

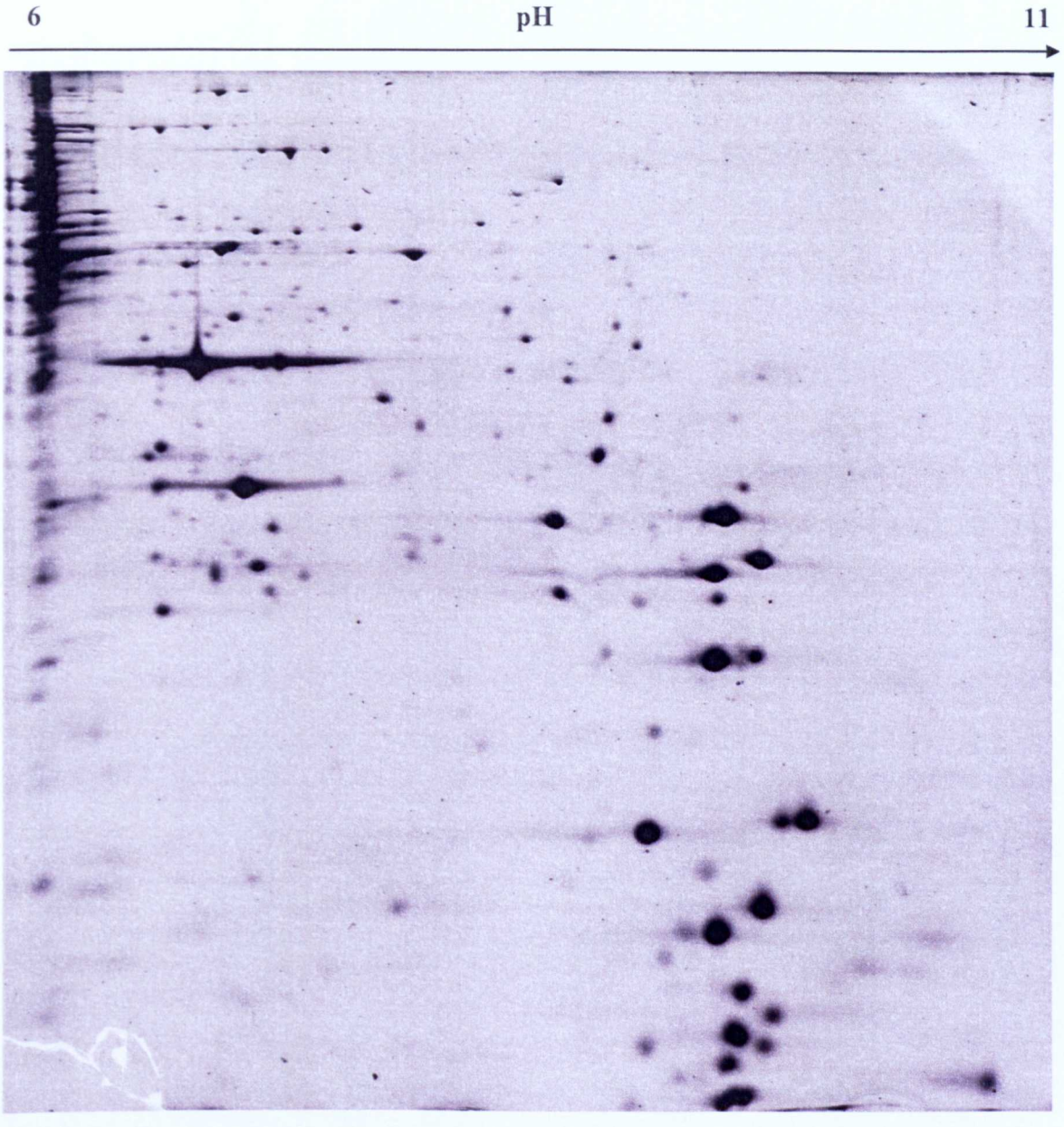

Figure 3.5 2DGE protein profile of J204 separated over a pH 6-11 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

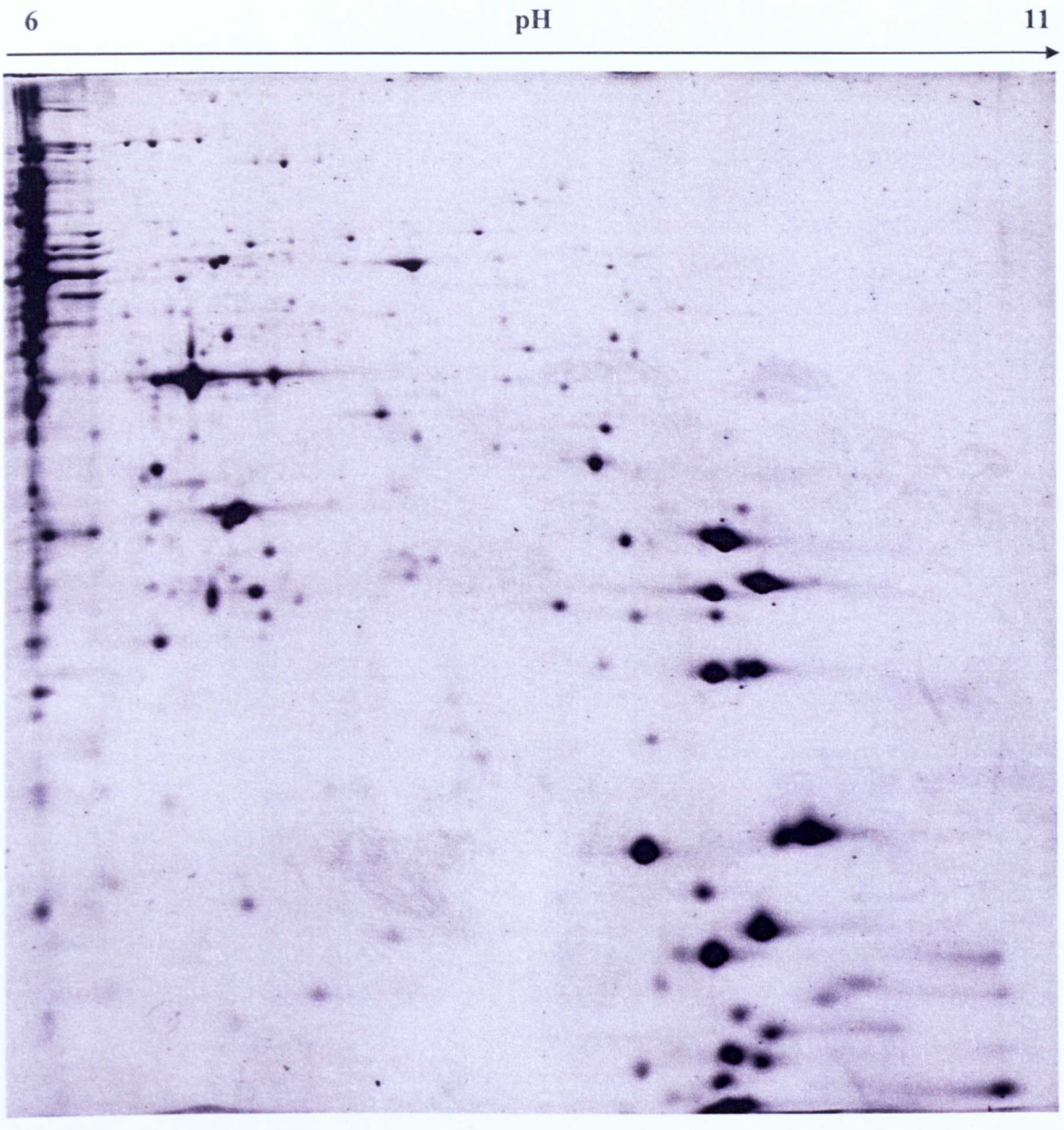

Figure 3.6 2DGE protein profile of J499 separated over a pH 6-11 gradient and on a 12% polyacrylamide gel. Proteins were stained with SYPRO ruby (Invitrogen) and visualised with an Ettan DALT imager (GE Healthcare).

3.3 Identifications of proteins excised from *E. coli* gels using MALDI-TOF MS

A small number of proteins were highlighted as present only in transformants, first by manual inspection and then confirmed with Proteomweaver software (version 3.0, Bio-Rad). These spots were manually excised, digested to peptides and desalted with Ziptips and submitted for MALDI-TOF analysis (refer to methods sections 2.16; 2.17; and 2.20). This was to see if 2DGE had achieved sufficient separation for protein identification and if any resistance enzymes could be detected. The spots that were predicted to be present in only one isolate and were identified through peptide mass fingerprints (PMFs) are displayed below in Figure 3.7 and described in Table 3.1.

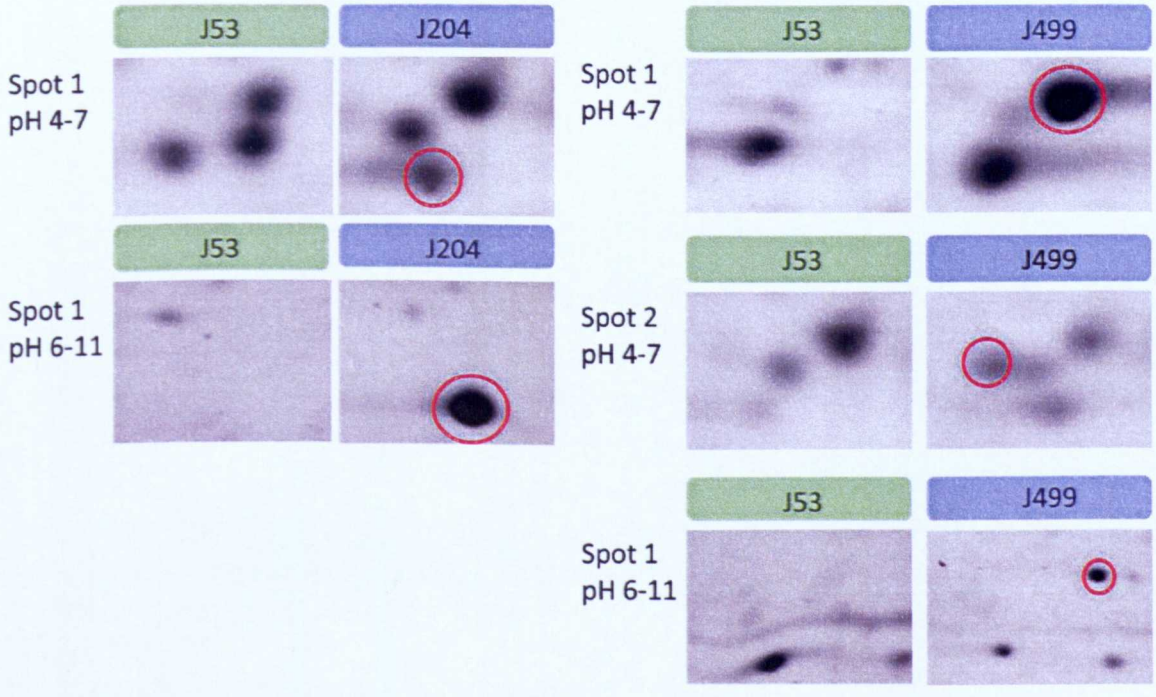

Figure 3.7 Protein spots thought to be expressed only in one isolate from transformant 2DGE protein profiles (circled in red).

The PMFs were searched against an in-house database consisting of all *E. coli* protein sequences downloaded from NCBI and the identifications returned are tabulated in Table 3.1.

Organism	Spot number	pH gradient	Protein Identification	Protein function	MASCOT Score
J204	Spot 1	4-7	TEM-1 precursor	TEM is a β-lactamase; it hydrolyses β-lactam antibiotics such as penicillin	79/67
	Spot 1	6-11	CTX-M ESBL	CTX-M hydrolyses extended-spectrum β-lactam antibiotics such as cefotaxime	70/67
J499	Spot 1	4-7	AAC(6)	AAC(6) acetylates aminoglycoside antibiotics and inactivates them	69/67
	Spot 2	4-7	TEM-1 precursor	TEM is a β-lactamase; it hydrolyses β-lactam antibiotics such as penicillin	190/67
	Spot 1	6-11	CTX-M ESBL	CTX-M hydrolyses extended-spectrum β-lactam antibiotics such as cefotaxime	81/67

Table 3.1 Identifications assigned to the protein spots that were present only in one of the transformants and not the others. MASCOT scores display the assigned score of the identification against the threshold value generated by MASCOT.

Although the CTX-M enzymes were successfully identified from the pH 6-11 profiles of the J53 transformants, presence or absence of plasmid-borne proteins can be assessed using other, more established methods *e.g.* PCR. To further elucidate the effects of plasmid acquisition, the more subtle changes in protein expression caused by acquisition of plasmids must be identified *i.e.* rather than the appearance/disappearance of spots, the proteins expressed by each isolate must be quantified. To achieve this, 2DGE was performed in triplicate and analysed by Proteomweaver and later SameSpots software. However, even in triplicate, no consensus of statistically significant changes could be reached due to variability between the gel profiles, highlighting a major reproducibility issue in 2DGE. To avoid this reproducibility issue, the 1D SDS-PAGE profiles of the transformants were analysed using the GeLC workflow (described in methods section 2.21). Prior to this, the phenotypes of the transformants were analysed to see if any differences could be identified, which might allow a more targeted approach to the GeLC proteome analysis. For instance, large differences in growth on glucose substrate could suggest protein differences in the pathways of carbon metabolism, while differences in osmotic tolerance may mean changes to the outer membrane protein complement.

3.4 Phenotypic analysis of CTX-M plasmid-bearing *E. coli*

As proteomic data describes but one aspect of resistance, additional approaches are required for a more in-depth analysis. For instance, a protein may not be expressed, but genome analysis is required to ascertain whether the gene is still present. Using a wider range of complementary techniques allows for validation of results from one technique against the others *e.g.* a proteome change that can be confirmed by a genome change allows for greater confidence in the interpretation of results. For this reason, the phenotypic characteristics of the transformant isolates compared with J53 were investigated. The phenotypic changes between the isolates could then be compared with any observed proteomic changes to allow a more comprehensive analysis of the effects of resistance plasmid procurement.

3.4.1 Compounds carried on PM plates

PM plates 1 and 2 contained substrates to investigate growth on organic metabolites and were thus used to infer any deficiencies in carbon metabolic pathways. PM plates 3 contained substrates to test for abnormalities in nitrogen metabolism, while plate 4 substrates investigated phosphorus and sulphur metabolism. PM plate 5 contained a variety of nutrient supplements such as amino acids, nucleic acids, vitamins metabolites and energy sources. PM plates 6, 7 and 8 all contained amino acids, di- and tripeptides to probe amino acid metabolism. PM plates 9 and 10 tested growth on a variety of osmotic challenges *e.g.* increasing NaCl concentrations, while the remaining PM plates 11 to 20 tested organism growth on a wide range of challenges, such as antibiotics, toxic compounds and anti-metabolites (See appendix 1 for a complete list of all compounds). All PM tests were carried out in duplicate.

3.4.2 Calculation of cut-off parameters

Numerical RA (respiration) values of growth were returned from the PM analysis and from this data, cut-offs were calculated for significant growth (more than the negative control) and stimulated growth (more growth than on the positive control) *e.g.* a metabolite has given the isolate an advantage, as in Methods section 2.4.2. These cut-offs are displayed in Table 3.2 and were used to calculate the cut-offs for significant *differences* in growth, given in Table 3.3.

Cut-offs for significant growth	**Isolates**		
	J53	**J204**	**J499**
Negative ctrl well PM1 A1	6668	6761	7679
Negative ctrl well PM2 A1	14348	10735	13901
Mean Average (avg)	10508	8748	10790
Standard Deviation (SD)	5430	2810	4399
Significant growth (avg + 1 SD)	15938	11558	15189
Parameter for significant growth (≥)	**16000**	**11500**	**15000**
Positive ctrl well PM1 C1 (D-glucose-6-phosphate)	30040	29296	27799
Positive ctrl well PM1 D9 (D-lactose)	31008	30275	26177
Mean Avg	30524	29785.5	26988
SD	684	692	1146
Avg + 1 SD	31208	30477	28134
Parameter for stimulated growth (≥)	**31000**	**30500**	**28000**

Table 3.2 Table describing how the PM cut-offs for significant (greater than the negative control well) growth and stimulated (greater than the positive control well) growth were calculated. Cut-off calculations were based on the method used by Morales *et al.* (Morales *et al.* 2005). Numbers correspond to RA (respiration) values generated by the Omnilog instrument.

Cut-offs for significant difference in growth	Comparison	
	J53 *vs.* J204	**J53 *vs.* J499**
Negative ctrl well PM1 A1	-93	1011
Negative ctrl well PM2 A1	3613	1359
Mean Avg	1760	1185
SD	2620	246
Significant growth (avg + 1 SD)	4380	1431
Parameter for significant growth (≥)	**4380**	**1430**

Table 3.3 Table describing how the cut-offs for the significant difference in growth between either J53 and J204 or J53 and J499. Cut-off calculations were based on the method used by Morales *et al.* (Morales *et al.* 2005). Numbers correspond to RA (respiration) values generated by the Omnilog instrument.

3.4.3 Results of PM analysis

The *E. coli* isolates were grown on PM plates for 48 hours as described in Methods section 2.4, time curves of their growth were recorded and are displayed in figures 3.8 and 3.9. J53 appeared to grow better on the amino acids and the di- and tri-peptides (PM plates 3-8), as well as the osmotic challenges (plates 9 and 10). While the transformants showed greater levels of respiration on many of the antimetabolite and antibiotic sensitivity plates (plates 12-20), this was expected for many substrates, given the resistance genes these plasmids encode. The carbon metabolism plates (PM 1 and 2) gave mixed results, with some substrates giving growth advantages to J53 and some to the transformants (see Figs. 3.8 and 3.9).

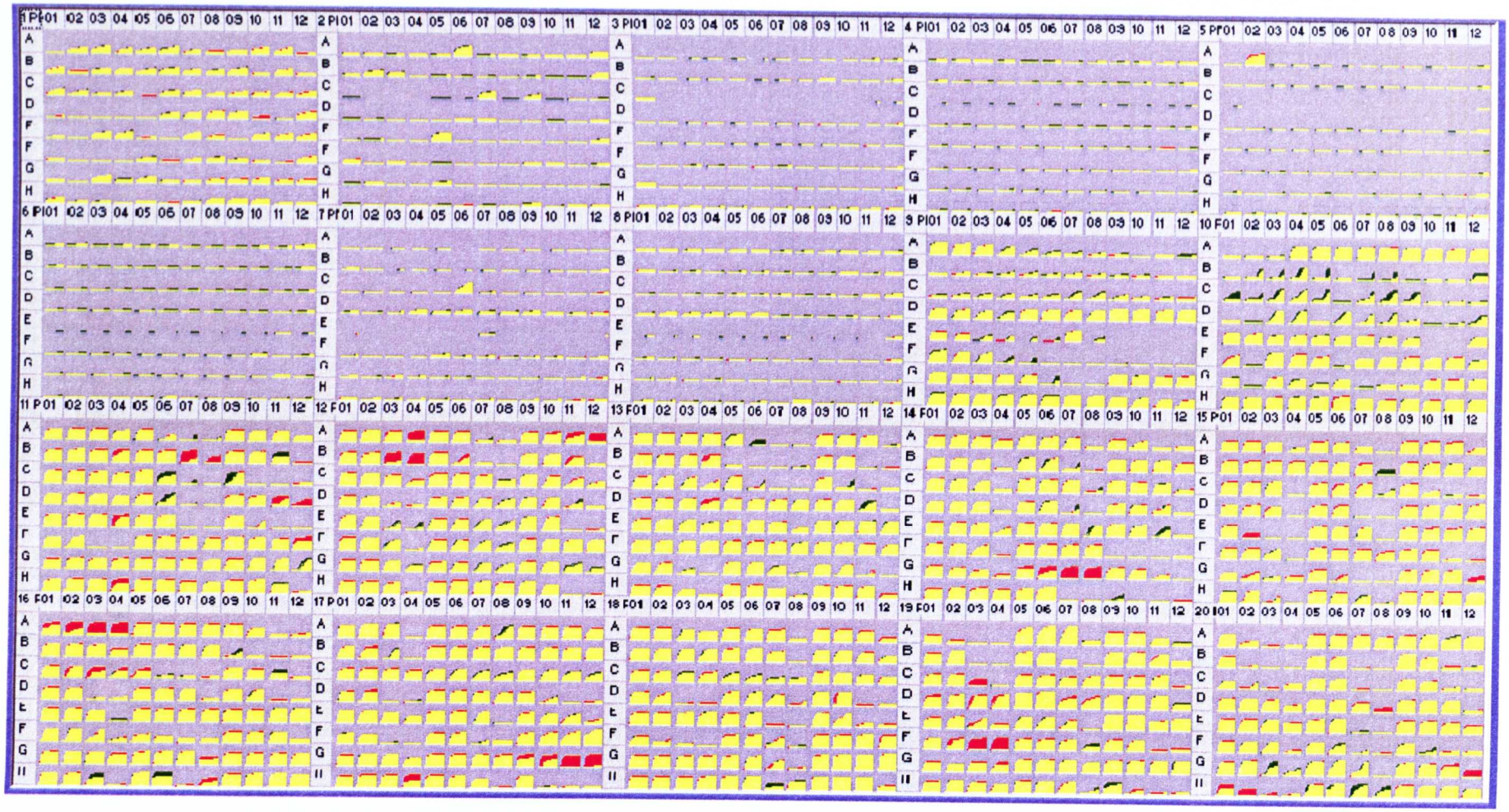

Figure 3.8 Graphic view of J53 and J204 growth over time, on all the substrates used in the twenty PM plates. Growth curves of J53 and J204 were overlayed and displayed; yellow is the equal growth of both isolates, green represents the growth of J53 only and red represents the growth of J204 only.

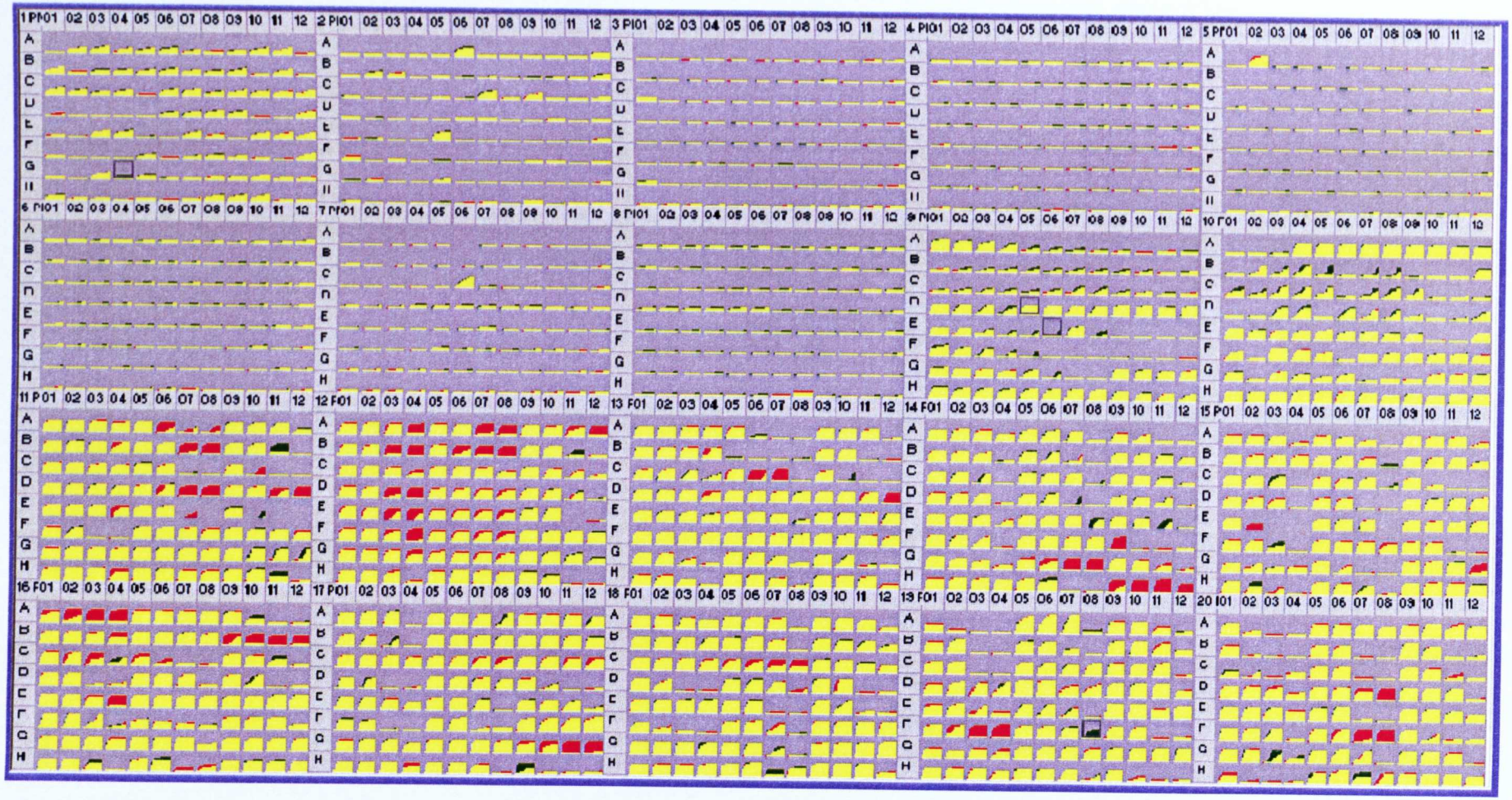

Figure 3.9 Graphic view of J53 and J499 growth over time, on all the substrates used in the twenty PM plates. Growth curves of J53 and J499 were overlayed and displayed; yellow is the equal growth of both isolates, green represents the growth of J53 only and red represents the growth of J499 only.

While there were many changes in growth between J53 and its transformant derivatives, the focus will only be on the changes which were statistically significant ($p < 0.05$, using Students' T-test), demonstrating greater than a 2-fold difference in growth and had no obvious resistance bias *e.g.* as the transformants both contained β-lactamases, all β-lactam substrates were removed from comparison with J53. These significant changes are displayed in Tables 3.4 and 3.5.

3.4.3.1 Similarities between plasmids

Both J204 and J499 showed a greater level of growth on the compounds: dichlofluanid, patulin and chloroxylenol, compared to J53. Dichlofluanid is an antifungal agent with antibacterial activity, often used as an anti-fouling agent to prevent attachment of organisms to a wetted surface, or biofouling (Fernandez-Alba *et al.* 2002). The activity stems from the ability to block thiol-containing enzymes involved in respiration (Leroux *et al.* 2010). The transformants had a particular advantage in the presence of this compound, both J204 and J499 displayed an 8-fold greater difference in RA values compared to J53.

Patulin is a mycotoxin produced by a variety of molds, most notably *Aspergillus* and *Penicillium*. It has antibacterial activity and may act as a quorum sensing inhibitor in *P. aeruginosa* (Liaqat & Thomas 2010), although its specific mechanism of action is unknown. J204 and J499 displayed differences in RA values of 5.4- and 4.8-fold greater than J53, respectively.

Chloroxylenol is an antibacterial drug with little toxicity to mammals and is present in antibacterial soaps such as Dettol. Its bactericidal activity is due to its ability to disrupt bacterial cell membrane potentials (Lear *et al.* 2002). J204 and J499 displayed differences in RA values of 3- and 2.7-fold greater than J53, respectively.

J53

PM type	Well	Test	Mode of Action	RA Difference in growth	*P* value (t-test)	Fold difference in growth *vs.* J204
PM09	A12	NaCl 10%	osmotic sensitivity	7708	0.0481	**2.24**
PM10	B05	pH 4.5 + L-Aspartic Acid	pH, decarboxylase	15504	0.0200	**2.48**
PM10	B07	pH 4.5 + L-Glutamine	pH, decarboxylase	9856.5	0.0264	**2.24**
PM10	C06	pH 4.5 + L-Tryptophan	pH, decarboxylase	5869	0.0054	**2.31**
PM11	A07	Chlortetracylcine	antibiotic	6008.5	0.0061	**2.22**
PM11	C07	Colistin	antibiotic	6475.5	0.0028	**4.80**
PM12	E04	2,4-Diamino-6,7-Diisopropylpteridine	Anti-vibrio agent, targets DHFR	10266	0.0081	**3.63**
PM13	A06	Dequalinium	ion channel inhibitor, K+ (m)	18720.5	0.0042	**16.93**
PM13	C10	Potassium chromate	toxic anion	9562	0.0106	**2.02**
PM14	H09	Sodium Orthovanadate	toxic anion, PO4 analogue	7665	0.0467	**2.49**
PM18	H07	2- Phenylphenol	DNA intercalator	17483	0.0235	**3.84**
PM20	F10	Pridinol	cholinergic antagonist	12200	0.0265	**2.30**

J204

PM type	Well	Test	Mode of Action	RA Difference in growth	*P* value (t-test)	Fold difference in growth *vs.* J53
PM15	G12	Menadione	respiration, uncoupler	-16887.5	0.0482	**2.08**
PM16	C03	Dichlofluanid	fungicide, phenylsulphamide	-26335	0.0036	**8.32**
PM16	H08	Chloroxylenol	fungicide	-15125	0.0052	**2.95**
PM20	H01	Patulin	antifungal, tubulin binding	-22530	0.0468	**5.38**

Table 3.4 Substrates that gave significant changes in growth (with $p < 0.05$, growth difference > 2-fold and no resistance bias) between isolates J53 and J204.

J53

PM type	Well	Test	Action	RA Difference in growth	*P*-value (t-test)	Fold difference in growth *vs.* J499
PM09	E08	3% Urea	osmotic sensitivity	12172	0.0105	**3.00**
PM10	B05	pH 4.5 + L-Aspartic Acid	pH, decarboxylase	14550	0.0033	**2.25**
PM12	B11	Polymyxin B	outer membrane	13829	0.0198	**2.32**
PM17	H09	Phenylarsine Oxide	tyrosine phosphatase	32762	0.0350	**2.84**
PM18	H07	2- Phenylphenol	DNA intercalator	20565.5	0.0323	**4.77**

J499

PM type	Well	Test	Action	RA Difference in growth	*P*-value (t-test)	Fold difference in growth *vs.* J53
PM01	D01	L-Asparagine	Carbon source	-6673	0.0373	**5.17**
PM14	F09	Sodium Metavanadate	transport, toxic anion, PO4 analog	-42975.5	0.0104	**13.34**
PM14	H09	Sodium Orthovanadate	transport, toxic anion, PO4 analog	-34842.5	0.0105	**2.29**
PM14	H10	Sodium Orthovanadate	transport, toxic anion, PO4 analog	-48136.5	0.0012	**14.60**
PM14	H11	Sodium Orthovanadate	transport, toxic anion, PO4 analog	-45530	0.0105	**10.21**
PM14	H12	Sodium Orthovanadate	transport, toxic anion, PO4 analog	-31444	0.0247	**5.61**
PM16	C03	Dichlofluanid	fungicide, phenylsulphamide	-26049	0.0032	**8.27**
PM16	H08	Chloroxylenol	fungicide	-12202	0.0368	**2.69**
PM20	H01	Patulin	Antifungal, tubulin binding	-17299.5	0.0416	**4.81**

Table 3.5 Substrates that gave significant differences in growth (with $p < 0.05$, growth difference > 2-fold and no resistance bias) between J53 and J499.

Conversely, 2-phenylphenol and the polymyxin antibiotics (polymyxin B and colistin) both gave a growth advantage to J53 over its transformant derivatives. The polymyxins act on the bacterial membrane, binding lipopolysaccharide which then allows disruption of the membrane and cell lysis. The susceptibility of the transformants to these agents suggests modification to the LPS/cell wall of J204 and J499, as 2-phenylphenol acts on DNA and would need entry to the cell for antimicrobial activity. J53 also displays increased growth during osmotic stress caused by 10% NaCl (*vs.* J204) and 3% urea (*vs.* J499), which also suggest changes to the cell wall which may affect cell permeability.

3.4.3.2 Effect of pEK204 on J53

The largest difference came when the isolates were grown on dequalinium, which gave J53 a 17-fold greater difference in RA values compared with J204. Dequalinium is a topical medicine and has been used in throat lozenges, mouthwashes and creams and ointments, although it is said to have lower activity against *E. coli* than against *S. aureus* due to the Gram-negative outer membrane (Tischer *et al.* 2012). Although many sites of action have been proposed for dequalinium, its exact mechanism is still unknown.

3.4.3.3 Effect of pEK499 on J53

The greatest difference between J53 and J499 RA values was from growth on vanadate ions, growth on metavanadate gave J499 a 13-fold greater difference in RA value compared to J53. Similarly high differences on the orthovanadate substrates were observed, the action of vanadate ions is thought to be through non-specific inhibition of ATPases. Growth in the presence of vanadate ions causes *P. aeruginosa* to alter it s LPS composition (Damron *et al.* 2012) and as J499 displayed such a difference in growth compared to J53, it suggests that J499 may have alterations to its cell envelope to cope with the vanadate stress.

3.5 GeLC analysis of *E. coli* extracts

Three biological replicates of J53, J204 and J499 protein extracts were separated by SDS-PAGE, the gel lanes cut into sections (Fig. 3.10.), digested to peptides and analysed with an orbitrap classic LC-MS/MS (see methods section 2.20). The resulting data was searched using Mascot (version 2.2.2, Matrix science) against an *E. coli* database, curated using all *E. coli* protein sequences downloaded from NCBI in August 2012. This identified all the peptides found in the digested gel fractions, rather than separating out proteins of interest and identifying them individually. The resulting Mascot .DAT files were analysed with Scaffold (version 3.3.2 Proteome Sciences) and in total, 767 proteins were identified between the isolates with a false discovery rate (FDR) of 0 %. Six hundred and ninety four of these proteins were identified in all three isolates, 16 were identified in both transformants, nine were identified in both J53 and J204, and eight were identified in both J53 and J499 (Fig. 3.10). The results also showed some proteins were identified in one isolate only: one was identified in J53, seven in J204 and 32 in J499 (Fig. 3.11).

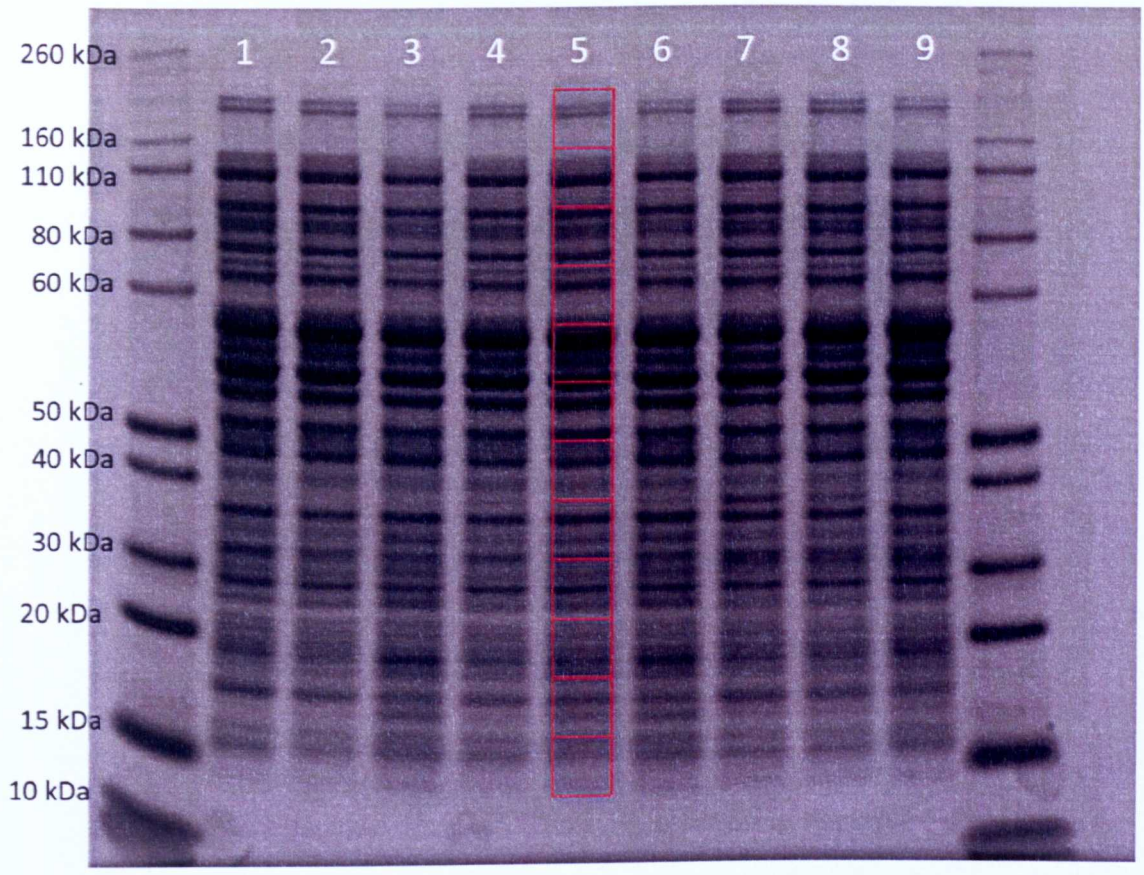

Figure 3.10. SDS-PAGE profiles of whole-cell extracts of the transformants. Three biological replicates of J53 (lanes 1, 2 and 3), J204 (lanes 4, 5 and 6) and J499 (lanes 7, 8 and 9) were run. Red ladder illustrates how protein profiles were divided and cut for GeLC analysis.

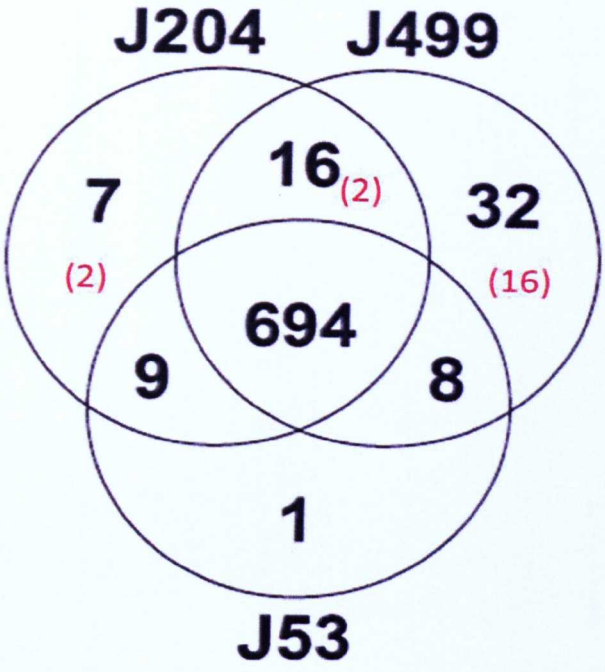

Figure 3.11 Venn diagram displaying the number of identified proteins shared between isolates or detected only in one isolate; generated by Scaffold software. Red numbers correspond to the number of plasmid-encoded proteins *e.g.* out of seven proteins identified only in J204, two were encoded by pEK204.

3.5.1 Identification of resistance plasmid proteins

The proteins identified from the GeLC profiles included TEM β-lactamase precursor and CTX-M-3 ESBL in both J204 and J499 (Table 3.8). It is understandable that the search algorithms used could not differentiate between the two different CTX-M enzymes carried by the two plasmids as they only differ by one amino acid (Poirel 2002) and the peptides with this difference may not have been detected. These are the only two resistance enzymes carried by pEK204 and this approach was able to identify them both. The resistance proteins: aminoglycoside N(6')-acetyltransferase AAC(6'), OXA-1 precursor, macrolide 2'-phosphotransferase I, DfrA17, aminoglycoside resistance protein and dihydropteroate synthase were identified as only expressed in J499. As well as the TEM and CTX-M proteins, this approach identified six out of the eight remaining resistance proteins on pEK499, a total of 8/10, leaving the tetracycline resistance protein (TetA) and chloramphenicol resistance protein (CatB4) undetected.

Isolate	Protein number	Protein Name	Gi number	Resistance gene?	No. of unique peptides
J499	1	Plasmid-partitioning protein SopA	gi|10955299	-	2
	2	Plasmid-partitioning protein	gi|10955300	-	2
	3	Dihydropteroate synthase	gi|111038084	Yes	3
	4	Conjugal transfer protein TraM	gi|116006876	-	3
	5	Conjugal transfer surface exclusion protein TraT	gi|149930813	-	4
	6	Plasmid segregation protein ParM	gi|157149363	-	4
	7	Stable plasmid inheritance protein PemK	gi|170650854	-	2
	8	Aminoglycoside resistance protein	gi|187736862	Yes	3
	9	DfrA17	gi|187736863	Yes	4
	10	Macrolide 2'-phosphotransferase I	gi|218707898	Yes	5
	11	Plasmid stable inheritance protein	gi|219586148	-	5
	12	Aminoglycoside N(6')-acetyltransferase	gi|256367629	Yes	6
	13	Beta-lactamase OXA-1 precursor	gi|256367630	Yes	4
	14	Putative HTH-type transcriptional regulator yfaX	gi|256367830	-	3
	15	Hypothetical protein pEK499_p136	gi|256367844	-	5
	16	Toxin-antitoxin system, toxin component, PIN family	gi|300820008	-	3
J204	17	Plasmid segregation protein ParM	gi|157149418	-	3
	18	Hypothetical protein EcE24377A_D0059	gi|157149438	-	2
J204 & J499	19	Beta-lactamase CTX-M-3 precursor	gi|256367540	Yes	9, 11
	20	Beta-lactamase TEM precursor	gi|169303001	Yes	6, 8

Table 3.6 List of plasmid-encoded proteins identified in transformants J204 and J499. Where two values of unique peptides were given, they correspond to J204 and J499 respectively, as differing numbers of peptides were found in each transformant.

This left 10 plasmid encoded proteins identified in J499, which were primarily involved in maintainance of pEK499 and included plasmid-partitioning protein SopA (Protein 1; Table 3.6), plasmid-partitioning protein (Protein 2; Table 3.6) which returned as SopB via BLASTp analysis (E = 0), conjugal transfer protein TraM (Protein 4; Table 3.6), conjugal transfer surface exclusion protein TraT (Protein 5; Table 3.6), plasmid segregation protein ParM (Protein 6; Table 3.6), stable plasmid inheritance protein PemK (Protein 7; Table 3.6), plasmid stable inheritance protein (Protein 11; Table 3.6) which returned as StbB via BLASTp analysis ($E = 2e^{-74}$), putative HTH-type transcriptional regulator yfaX (Protein 14; Table 3.6), hypothetical protein pEK499_p136 (Protein; Table 3.6) and toxin-antitoxin system, toxin component, PIN family (Protein 16; Table 3.6).

There were two proteins from J204 which were expressed from the plasmid but not involved in resistance. These were plasmid segregation protein ParM (Protein 17; Table 3.6) and hypothetical protein EcE24377A_D0059 (Protein 18; Table 3.6) which returned as plasmid mobilisation protein MobC ($E = 2e^{-71}$).

In total, this left five proteins identified in J204 and 16 proteins identified in J499 which were not plasmid-encoded and where expression was likely to be induced only after plasmid acquisition (as these proteins were not detected in J53).

3.5.2 Effects of plasmid acquisition on the host *E. coli* strain J53

3.5.2.1 Non-plasmid encoded proteins induced or repressed by plasmid acquisition

The single protein identified only in J53 (Fig. 3.11) was periplasmic TolA-binding protein (Table 3.7) and returned as YbgF by BLASTp analysis (E = 0), a co-regulator of the Tol-Pal system required for membrane integrity and which participates in the septation process during cell division (Krachler *et al.* 2010). YbgF is not required for Tol-Pal functional activity and although YbgF is known to bind TolA, to date the exact function is unknown.

Isolate	Protein number	Protein Name	Gi number	No. of unique peptides
J499	1	Bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino)uracil reductase	gi|110640675	2
	2	Thymidylate kinase	gi|110641274	2
	3	Hypothetical protein ECP_1864	gi|110642036	2
	4	Lipoprotein YfhM	gi|110642685	2
	5	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	gi|110643429	2
	6	Protease TldD	gi|110643479	2
	7	Hypothetical protein ECP_3589	gi|110643736	2
	8	Lysine decarboxylase, inducible	gi|110644490	3
	9	Hypothetical protein ECP_4483	gi|110644595	2
	10	*ubiD* gene product	gi|117626121	2
	11	Exodeoxyribonuclease VII small subunit	gi|157155184	2
	12	tRNA-specific 2-thiouridylase MnmA	gi|157156703	2
	13	Hydroxyacylglutathione hydrolase	gi|157159673	2
	14	Hypothetical protein EcHS_A4153	gi|157163396	2
	15	Hypothetical protein APECO1_O1CoBM79	gi|157418162	2
	16	Hydrolase, alpha/beta fold family	gi|191173631	5
J204	17	Dihdropyrimidine dehydrogenase	gi|110642356	2
	18	Threonine dehydratase	gi|110643363	2
	19	Quinone oxidoreductase	gi|110644386	2
	20	Cobyrinic acid a,c-diamide synthase	gi|226201035	2
	21	ATP-dependent protease ATP-binding subunit	gi|32141212	2
J53	22	Periplasmic TolA-binding protein	gi|16128717	2

Table 3.7. Proteins identified in only one isolate and that were not encoded by a resistance plasmid.

There were seven proteins identified only in J204 including two proteins which were identified as originating from the plasmid pEK204. The remaining five proteins (Table 3.7) included Dihdropyrimidine dehydrogenase (Protein 17; Table 3.7) which is required for uracil catabolism. Threonine dehydratase (Protein 18; Table 3.7) is involved in amino acid metabolism, while Quinone oxidoreductase (Protein 19; Table 3.7) participates in electron transfer in respiration. Cobyrinic acid a,c-diamide synthase (protein 20; Table 3.7) is involved in the synthesis of vitamin B_{12}, while protein 21 returned as ClpB by BLASTp analysis (E = 0), involved in disaggregation and refolding of proteins rather than degradation (Zolkiewski 2006).

The 32 proteins identified only in J499 included 16 proteins which originated from the plasmid pEK499. The remaining 16 proteins (Table 3.7) included Bifunctional diaminohydroxy-phosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino) uracil reductase (Protein 1; Table 3.7), which returned as RibD via BLASTp analysis (E = 0) and is involved in the biosynthesis of riboflavin. Thymidylate kinase or Tmk (Protein 2; Table 3.9) is an essential enzyme catalysing the synthesis of Thymidine deoxynucleotide precursors. UDP-N-acetylglucosamine 1-carboxyvinyltransferase or MurA (Protein 5; Table 3.7), this protein has been highlighted as a potential resistance determinant for fosfomycin with a low fitness cost (Couce *et al.* 2012).

TldD (protein 6, Table 3.7) is known to be involved in the control of DNA Gyrase regulation and may play a role in Ccd toxin addiction systems (Allali *et al.* 2002), which would explain the presence of this protein in J499 (pEK499 encodes a Ccd addiction system).

Hypothetical protein ECP_3589 (Protein 7; Table 3.7) returned as YhiR via BLASTp analysis (E = 0) and is involved in the methylation of rRNA.

Lysine decarboxylase, inducible (Protein 8; Table 3.7) is involved in protection against acid stress and also the stringent response, though to regulate lysine metabolism under nutrient-limiting conditions (Kanjee *et al.* 2011).

The *ubiD* gene product (Protein 10; Table 3.7) is involved in the synthesis of coenzyme Q, required for the periplasmic oxidizing system in removing electrons via the electron transport system (Gulmezian *et al.* 2008).

tRNA-specific 2-thiouridylase MnmA (Protein 12; Table 3.7), the Mnm proteins are involved in the modification of tRNA (Armengod *et al.* 2012).

Neither hypothetical proteins; hypothetical protein EcHS_A4153 (Protein 14; Table 3.7) and Hypothetical protein APECO1_O1CoBM79 (Protein 15; Table 3.7) returned any definitive matches by BLASTp analysis.

Hydrolase, alpha/beta fold family (Protein 16; Table 3.7) returned as X-Pro dipeptidyl-peptidase via BLASTp analysis (E = 0), which cleaves any dipeptides containing proline. As there was little further information on the functions and activities of the remaining proteins, their role in this resistance mechanism is unknown.

3.5.3 Proteins found only in two isolates

3.5.3.1 Proteins shared by transformants

There were sixteen proteins identified as present in both transformants and not in J53, these included TEM-precursor and CTX-M-3 as mentioned in section 3.5.1, these and the remaining 14 are displayed in Table 3.8. Aside from the proteins 1 and 2 (Table 3.8), there were no additional proteins identified as expressed from the plasmids pEK204 and pEK499. Other proteins identified included: hypothetical protein ECP_2945 (Protein 3; Table 3.8) which returned as YggS by BLASTp analysis ($E = 1e^{-170}$).

Translocation protein TolB (Protein 4; Table 3.9) is a major constituent of the Tol-Pal system mentioned in section 3.5.2.1, TolB works with the other members of the Tol-Pal system to preserve membrane integrity and organise the septation process of cell division. Deletion of any tol genes results in non-functional membrane (leaking, reduced LPS) and sensitivity to large antibiotics and detergents (Bonsor *et al.* 2009).

Peptidoglycan-associated outer membrane lipoprotein returned as the *pal* gene product by BLASTp analysis ($E = 5e^{-123}$), Pal is also part of the Tol-Pal system required for membrane integrity and which participates in the septation process during cell division (Krachler *et al.* 2010).

Protein number	Protein Name	Gi number	No. unique peptides
1	Beta-lactamase CTX-M-3 precursor	gi|256367540	9, 11
2	Beta-lactamase TEM precursor	gi|169303001	6, 8
3	Hypothetical protein ECP_2945	gi|110643100	2, 2
4	Translocation protein TolB	gi|110640948	2, 2
5	Peptidoglycan-associated outer membrane lipoprotein	gi|110640949	2, 2
6	Nitric oxide dioxygenase	gi|110642714	2, 2
7	23S rRNA methyltransferase	gi|110643419	2, 2
8	ABC transporter ATP-binding protein	gi|110640346	2, 2
9	6-phospho-beta-glucosidase	gi|110643050	2, 2
10	3,4-dihydroxy-2-butanone 4-phosphate synthase	gi|110643290	2, 2
11	Ribonuclease III	gi|110642729	2, 2
12	D-ribose transporter ATP-binding protein	gi|110644090	2, 2
13	*obgE* gene product	gi|386602734	2, 2
14	N-methyltryptophan oxidase, FAD-binding	gi|16129022	2, 2
15	RNA-binding protein YhbY	gi|110643420	2, 2
16	DNA-binding/iron metalloprotein/AP endonuclease	gi|110643308	2, 2

Table 3.8. Proteins identified in both transformants J204 & J499, but not in J53. Two values are given for the unique peptides, corresponding to J204 and J499 respectively, as differing numbers of peptides were found in each transformant.

Nitric oxide dioxygenase (protein 6; Table 3.8), which returned as HmpA or flavoheamoprotein by BLASTp analysis (E = 0), it is involved in the tolerance of reactive nitrogen intermediates (RNIs) and is utilised by pathogenic bacteria, including ExPEC (Bateman & Seed 2012).

3,4-dihydroxy-2-butanone 4-phosphate synthase (Protein 10; Table 3.8) returned as RibB via BLASTp analysis ($E = 5e^{-157}$) and is involved in flavin biosynthesis.

Ribonuclease III (Protein 11; Table 3.8) is an endonuclease that produces functional RNAs such as ribosomal RNA from cleaving its precursor (Macrae & Doudna, 2007).

ObgE (Protein 13; Table 3.8) or CgtA, is an essential GTPase in *E. coli* and has been implicated in the control of the stringent response in response to amino acid starvation (Persky *et al.* 2009).

DNA-binding/iron metalloprotein/AP endonuclease (Protein 16; Table 3.8) returned as YgjD via BLASTp analysis (E = 0) and is involved in tRNA modifications but may also have glycoprotease activity (Hashimoto *et al.* 2011).

Very little further information was obtained for the remaining proteins, therefore their potential role in this resistance mechanism is currently unknown.

3.5.4 Proteins shared by J53 and one transformant

Nine proteins were identified as expressed in J53 and J204 but not in J499, these included: FtsH protease regulator HflC (Table 3.9) regulates the protease FstH (HflB), which re-folds misfolded proteins. HflC also protects the bacteriophage protein CII from degradation, which promotes the lysogenic cell cycle (Bandyopadhyay *et al.* 2010).

The *mtlA* gene product (Protein 2; Table 3.9) returned as the mannitol-specific EIIA subunit of the phosphotransferase system (PTS) (E = 0), which catalyses the transport and concomitant phosphorylation of sugars, in this case, mannitol (Kumar *et al.* 2011).

Protein 3 (Table 3.9) returned as MppA by BLASTp analysis (E = 0), MppA is a peptide permease which also has haem-binding activity and hence iron regulation. To utilise haem, *E. coli* must express either DppA or MppA (Letoffe *et al.* 2006).

The *wzzB* gene product (Protein 4; Table 3.9) acts as a regulator of polysaccharide chain length in the biosynthesis of lipopolysachaarides (LPS), which can affect the properties of LPS (Woodward *et al.* 2010).

Protein 7 (Table 3.9) returned as *yrbD* gene product by BLASTp analysis (E = $3e^{-128}$), a toluene transport protein which has been implicated as an immunogenic protein of *Y. pestis* (Tanabe *et al.* 2006).

As in the previous section, little further information was obtained for the remaining proteins, therefore their potential role in this resistance mechanism is currently unknown.

Isolates	Protein number	Protein Name	Gi number	No. unique peptides
J53 and J204	1	FtsH protease regulator HflC	gi|110644532	2, 2
	2	*mtlA* gene product	gi|15804140	2, 2
	3	bacterial extracellular solute-binding protein, family 5	gi|300822500	2, 3
	4	*wzzB* gene product	gi|15802506	2, 2
	5	glucose-1-phosphate adenylyltransferase	gi|110643671	2, 2
	6	aromatic amino acid aminotransferase	gi|157163523	2, 2
	7	hypothetical protein c3953	gi|26249779	2, 2
	8	2-hydroxyacid dehydrogenase	gi|110641209	2, 2
	9	2,5-diketo-D-gluconate reductase B	gi|110640422	2, 2
J53 and J499	10	S-formylglutathione hydrolase	gi|16128340	2, 3
	11	sigma(54) modulation protein	gi|110643444	2, 2
	12	co-chaperone HscB	gi|110642692	2, 2
	13	malate synthase G	gi|157162448	2, 3
	14	fructokinase	gi|293413645	2, 2
	15	oxidoreductase YgjR	gi|110643331	2, 2
	16	competence damage-inducible protein A	gi|110642820	2, 2
	17	Peroxide resistance protein, lowers intracellular iron	gi|16128000	2, 3

Table 3.9. Proteins identified in both J53 and one of the transformants but not the other. Two values of unique peptides were given for J53 and the transformant respectively, as differing numbers of peptides were found in each isolate.

There were also eight proteins identified as expressed in J53 and J499, but not in J204 and include: S-formylglutathione hydrolase (Protein 10; Table 3.9), required for detoxification of formaldehyde. Sigma (54) modulation protein (Protein 11; Table 3.9) returned as Yhbh by BLASTp analysis (E = $3e^{-62}$) which promotes and stabilises 100S ribosome formation during the transition to stationary phase growth (Ueta *et al.* 2005). 100S ribosomes are 70S dimers and have no translational activity, they are thought to protect against ribosomal degradation, resulting in a longer cellular lifespan.

Co-chaperone HscB (Protein 12; Table 3.9) is required for transfer of Fe-S clusters into proteins (Ciesielski *et al.* 2012). Proteins 13, 14 and 15 (Table 3.9) were involved in metabolic processes but the reasons for their presence in J53 and J499, but not in J204 are unknown.

Competence damage-inducible protein A or YgaD returned as similar to CinA by BLASTp analysis (E = $1e^{-114}$), CinA is thought to be required for the process of transformation as expression is required for competence (Luo & Morrison 2003).

Peroxide resistance protein returned as YaaA by BLASTp analysis (E = 0), it acts to reduce hydrogen peroxide toxicity through suppression of unincorporated intracellular iron levels (Liu *et al.* 2011).

3.6 Chapter Summary

This investigation into MDR plasmid acquisition (pEK204 and pEK499) in *E. coli* sought to characterise the proteins changes caused by uptake of these plasmids. The aim was to use the PM results to corroborate with the proteomics results to confirm any changes identified and try to elucidate why these changes may have occurred. 2DGE was used to separate the proteins from a whole-cell extract and identify the digested proteins with MALDI-TOF MS. While useful in this case to identify single proteins expressed from the transformants, 2DGE is not an applicable technique for a high-throughput reference laboratory, as it generally requires analysis of multiple isolates and is a lengthy technique. Also, the approach only identifies single proteins, whereas the GeLC approach is more suitable and has the potential to profile the expressed resistance proteins. Although the GeLC method was not the optimal approach to identify all the proteins on the plasmid (compared with *e.g.* sequencing), it was certainly an effective method to ascertain which proteins

were actually expressed from the plasmid. Eight of the ten resistance proteins from pEK499 were detected in the J499 protein extract and both the resistance proteins from pEK204 were detected. The two remaining resistance proteins from pEK499 were tetracycline resistance protein Tet(A) and chloramphenicol resistance protein CatB4, however, the reasons why these proteins were not detected are unknown. This approach therefore has the potential to identify proteins in an extract, as well as to profile the resistance proteome expressed by a resistant isolate. This is a technique that would be more applicable to a reference laboratory, as it is high-throughput, sensitive and simple sample preparation (however, an LC-MS/MS is still required). Due to its rapid timescale and global coverage of so many phenotypes, the Biolog system could also be utilised in a reference laboratory, but at a reduced scale. There are too many plates which give information that is not relevant when investigating resistance phenotypes *e.g.* aside from MIC testing, very few phenotypic/biochemical tests are ever utilised for investigation in resistance laboratories.

PM analysis demonstrated that pEK204 provided J204 with an advantage on dequalinium, for which resistance is usually conferred by efflux pumps (Turner *et al.* 1997; Korkhov & Tate 2008). The acquisition of pEK204 may have caused the upregulation of one or more efflux proteins in J53. PM analysis also showed that pEK499 provided J499 with high tolerance to vanadate ions (more than a 10-fold difference in growth compared to J53). Vanadate ions inhibit ATPase activity (Matsuo *et al.* 2008), so pEK499 may confer J53 with some way of overcoming this inhibition, either through increased expression of the targets, or expression of an additional ATPase to quench the vanadate ions. However, even after proteomic analysis, the precise reasons for these substrate advantages are unknown.

Both the transformants could grow on higher levels of patulin, dichlofluanid and chloroxylenol than their parent J53. Dichlofluanid, chloroxylenol and patulin to a lesser extent are all present in the environment, Dettol (of which chloroxylenol is an active ingredient) is used in hospitals and homes and dichlofluanid is used around watery areas *e.g.* rivers and ports. These plasmids could provide the recipient with a slight survival advantage compared with bacteria without these phenotypic changes which may allow ex vivo survival in human-populated areas *e.g.* places of rest/work and the water system. This may contribute to the plasmids' dissemination and

prolonged carriage and could help to explain why in the absence of antibiotic selection pressure, these MDR plasmids are kept by the bacteria. This is a logical viewpoint as bacteria use the same general mechanisms for biocide and antibiotic resistance (Sheldon 2005). Indeed, it is known that exposure to biocides may select for antibiotic resistance (Gilbert & Mcbain 2003), but this result suggests (but does not confirm) that the opposite may be true, that antibiotic resistance could also select for reduced biocide susceptibility.

It was demonstrated that plasmid acquisition did confer some disadvantages on some of the compounds tested, as PM analysis showed that J53 displayed more growth on polymyxin antibiotics (polymyxins B and colistin), 2-phenylphenol and displayed greater osmotic tolerance than both transformants (by growing better on both urea and sodium chloride). Combined with the advantages the plasmids appear to give, the data suggests that the transformants undergo some modulation of the cell envelope. For instance, the polymyxins act on the cell membrane and show greater activity against transformants and 2-phenylphenol has an intracellular target, so it must be more able to permeate the membrane.

The proteomics results support this general hypothesis, as proteins novel to J204 and J499 and to J53 included membrane proteins. In particular, plasmid acquisition seemed to cause modulation of the Tol-Pal system, required for membrane structural integrity (Krachler *et al.* 2010) *e.g.* a modulator of TolA (FhgY) was identified only in J53, whereas TolB and Pal were identified in only J204 and J499. This could explain the differences in the organisms' phenotype as deletion of any *tol* genes can result in a non-functional membrane (leaking, reduced LPS) and sensitivity to large antibiotics (such as peptide antibiotics) and detergents (Bonsor *et al.* 2009). Therefore, if plasmid acquisition did affect FhgY levels, this may have compromised membrane functionality and led to increased sensitivity to certain antibiotics *e.g.* polymyxins. It should be noted that although a protein was identified in two isolates and not the other one, it may not necessarily suggest that this protein was not expressed, rather it may have been at levels too low to detect. Peptides could have been sampled but fragmented insufficiently or simply the stochastic manner of data dependent acquisitions could have lead to the missing data.

Additional proteins identified only in the transformants included HmpA, which protects against RNI and is utilised by pathogenic bacteria in stress defence. Also identified was ObgE, which modulates the stringent response to amino acid starvation and is involved in DNA repair through stimulation of *recA* (Zielke *et al.* 2003). From the proteomic data, it appeared that these proteins were not expressed in J53 and they could be advantageous to the transformants under conditions of stress, such as antibiotic treatment. They could therefore be responsible for the unexplained effects of plasmid acquisition *e.g.* increased tolerance to antiseptics, due to their stress defence functions, although further work is required to confirm the role of these proteins. There were also many differences between proteins expressed in J53 and J204 compared to J53 and J499. The reasons for expression of these proteins in one transformant and not the other are as yet unclear, as they do not correlate with the phenotypic differences.

Proteins have been identified which were expressed or repressed upon acquisition of MDR plasmids, some of which may contribute to the altered susceptibilities of the transformants. It is recognised that although plasmids may not encode enzymes directly responsible for resistance to antibacterial agents, plasmid acquisition can change the resistance profile of an organism. For instance, certain R plasmids were found to reduce the levels of expressed OmpF in *E. coli*, which reduced susceptibilities to many other agents (Rossouw & Rowbury 1984). Russell also highlights examples where plasmid acquisition has altered cell envelope composition (Russell 1997), corroborating with the results in this study, that acquisition of MDR plasmids can alter the proteome and phenotype of the host organism. Also, different MDR plasmids can produce subtly different phenotypes and protein profiles, so further testing of a wide variety of plasmids on the same organism (J53) is required.

The proteomic experiments undertaken in this chapter have identified many proteins and subsequently highlighted areas which require further investigation. More experimentation needs to be carried out on the proteins identified in this study to further characterise their precise role in modulation of host cell proteome upon acquisition. For instance, mutants lacking these proteins should be generated and tested again to see if susceptibilities have changed. Further work also needs to be done on the susceptibilities of transformants to biocides and antiseptics, because,

although there are no specific resistance genes on the plasmids for tolerance to antiseptics, the transformants clearly have an advantage in the presence of certain agents.

4. Results
Carbapenem resistance in *Klebsiella pneumoniae*

4.1 Background of Isolates

A well-described mechanism of resistance to carbapenems is through expression of a metallo-β-lactamase or carbapenemase enzyme. These include KPC from *Klebsiella* sp. (Nordmann *et al.* 2009), OXA-type carbapenemases from *e.g. A. baumannii* (Woodford *et al.* 2006), IMP and VIM enzymes from *P. aeruginosa* (Livermore & Woodford, 2006) and the notorious NDM-1 from *E. coli* and *K. pneumoniae* (Karthikeyan *et al.* 2010). However, other resistance mechanisms may confer carbapenem resistance, such as reduced porin expression in combination with AmpC or ESBL enzymes and upregulated efflux-pumps. Altered porin expression and the effects of outer membrane protein rearrangement on the organism is of particular interest, as isolates displaying altered porin expression have MICs similar to carbapenemase-producers but will return negative PCR results for carbapenemases.

Due to the rapid inter-species dissemination of plasmid-mediated carbapenemases *e.g.* KPC enzymes, originally from *Klebsiella pneumoniae* (Queenan & Bush, 2007), carbapenem-resistant organisms are an increasing healthcare concern as this resistance eliminates the agents of last resort for many Enterobacteriaceae. Hence, carbapenem-resistant *K. pneumoniae* are associated with fewer treatment options, namely combination therapies using tigecycline with polymyxins and increased mortality (40-50%) (Qureshi *et al.* 2012).

The organisms used in this study consist of a pre- and post therapy pair of *K. pneumoniae* clinical isolates, 1A and 1B, respectively. 1A was recovered from a patient receiving piperacillin/tazobactam and gentamicin and upon isolation, treatment was changed to meropenem. Four weeks later a second carbapenem-resistant *K. pneumoniae* isolate, 1B, was recovered and while both isolates were PCR-negative for carbapenemases, they tested positive for a group 1 CTX-M ESBL. The deduced mechanism of resistance was porin reduction combined with ESBL production.

The aims of this study are to characterise changes in *K. pneumoniae* proteome caused by differential porin expression. For instance, it is worth investigating whether any other OMP expression is affected by the potential reduction of two major porins. Or, if there is a potential

target to circumvent this mechanism of resistance and more importantly, whether there are potential markers which may be used to speed up the detection of this resistance mechanism.

4.2 Membrane fractionation and 1D gels of *K. pneumoniae*

Initially, to check the suspected differential porin expression, the OMPs from 1A and 1B were analysed using SDS-PAGE. Proteins extraction methods were optimised using a variety of OMP fractionation methods and the ROMP method proved fastest and gave good OMP separation (see methods section 2.7.2). The OMP extracts were run on two different SDS-PAGE systems, one was cast in-house and the other was the Nu-PAGE bis-tris gel system supplied by Invitrogen (Fig. 4.1). It was determined that the in-house gels gave improved porin resolution compared with the Nu-PAGE gels, so they were used for this preliminary experiment.

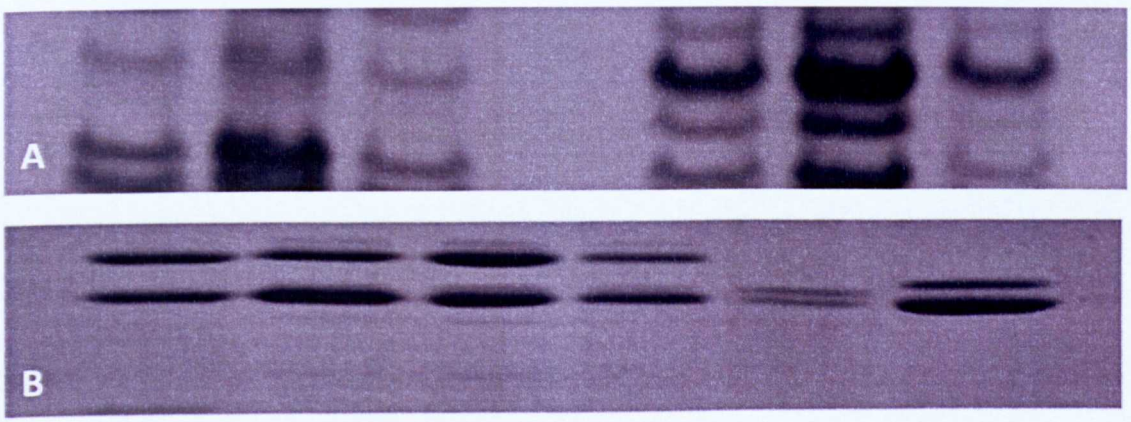

Figure 4.1. Comparison of two types of SDS-PAGE used to run OMP protein extracts, shown here are the bands between 30 and 40 kDa: NuPAGE gels (A) were purchased from Invitrogen while gels cast in-house (B) gave preferred separation of OMPs.

The OMP fractions of the paired isolates 1A and 1B were run alongside a pair of carbapenem-susceptible *K. pneumoniae*, isolate K2, a control for porin loss lacking OmpK35 and OmpK36 and the *K. pneumoniae* type strain ATCC 13883 (NCTC 9633) for a full OMP complement (Fig. 4.2). From the OMP profiles of the gel, it was clear that while isolate 1A had

similar expression to the other carbapenem-susceptible isolates, 1B had lost two bands at roughly 38-40 kDa and reduced expression of the remaining bands at 36.5 kDa. This pattern is similar to that of isolate K2, which lacked expression of two major porins OmpK35 and OmpK36 (Doumith *et al.* 2009) and confirmed the suspected carbapenem-resistance mechanism mediated by porin loss and ESBL-production (Webster *et al.* 2010).

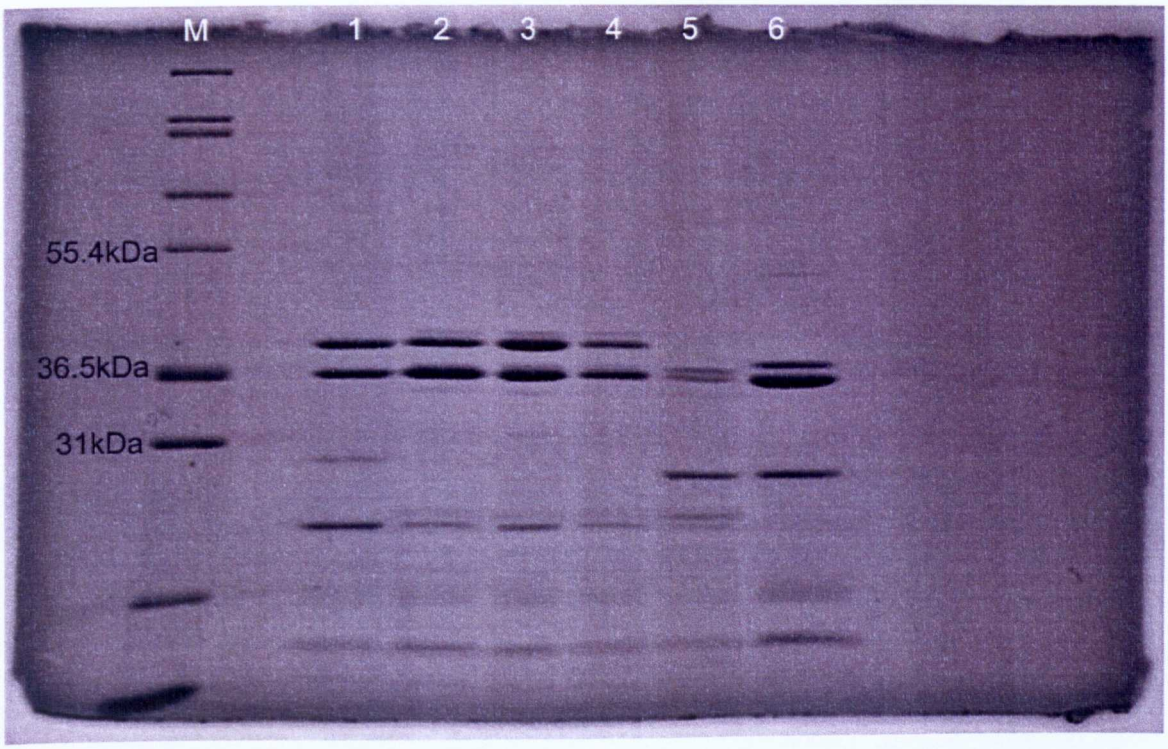

Figure 4.2. In-house SDS-PAGE profiles of *K. pneumoniae* OMP fractions. Proteins were run on a 12% polyacrylamide gel with **M)** marker, **1)** ATCC 13883 **2/3)** a pair of carbapenem-susceptible *K. pneumoniae* isolates **4)** isolate 1A **5)** isolate 1B **6)** *K. pneumoniae* isolate K2, which is lacking OmpK35 and OmpK36 expression.

4.3 GeLC analysis of *K. pneumoniae* outer membrane proteins

As SDS-PAGE only provides information on the presence or absence of a band and no identifications are assigned, the OMP fraction from *K. pneumoniae* isolates were subjected to GeLC analysis. The protein profile from SDS-PAGE was cut into 12 pieces (Fig. 4.3), digested with trypsin and submitted for LC-MS/MS analysis. The SDS-PAGE added a preliminary layer of separation to the complex mixture of proteins prior to LC peptide separation and allowed greater resolution of peptides for improved protein identification.

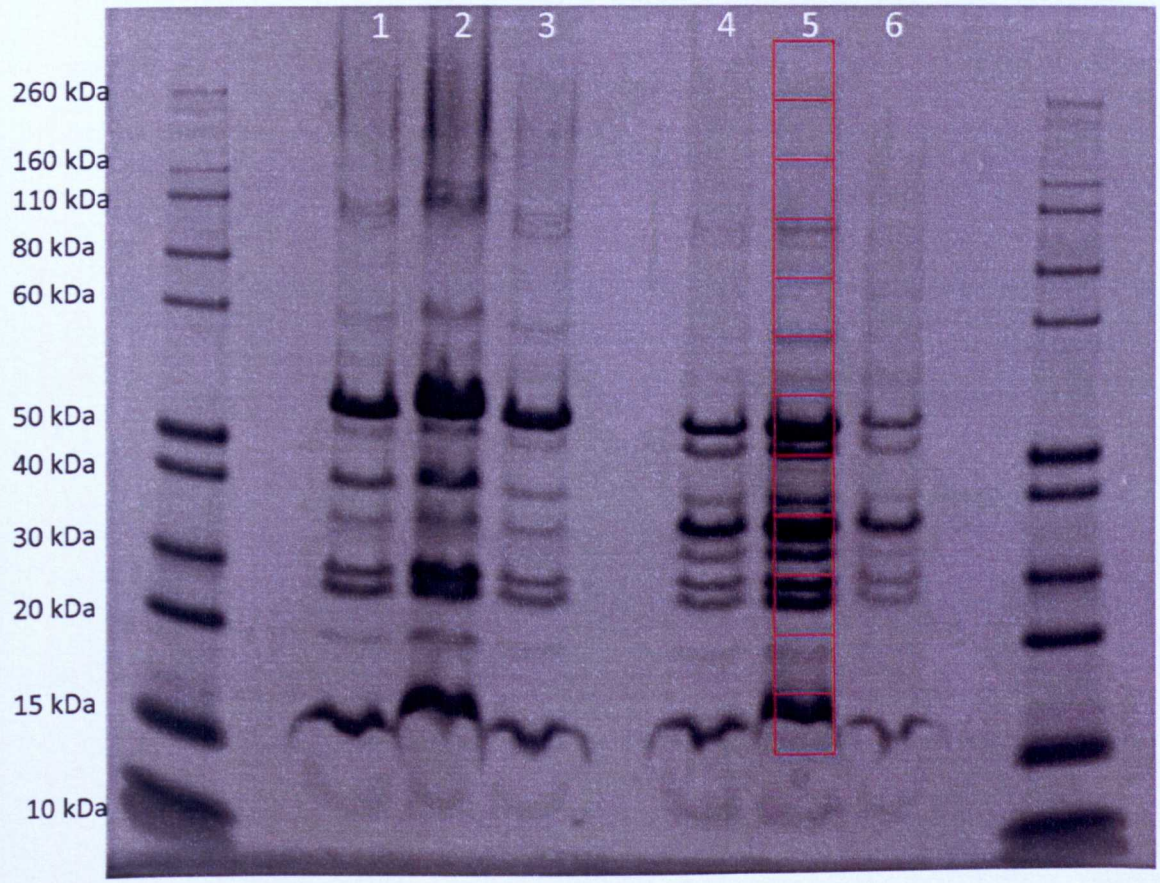

Figure 4.3. Invitrogen SDS-PAGE profiles of OMP extracts of the *K. pneumoniae* clinical pair. Three biological replicates of isolate 1A (lanes 1, 2 and 3) and isolate 1B (lanes 4, 5 and 6) were run. Red ladder illustrates how protein profiles were divided and cut for GeLC analysis.

The raw MS spectra output files were first subjected to peptide matching, against a protein sequence database using Mascot (version 2.2.2, Matrixscience). The database used was curated in-house using sequences of all *Klebsiella* sp. protein sequences obtainable from NCBInr (August 2012). The resulting .DAT files were analysed with Scaffold software (version 3.6, Proteome sciences) as detailed in methods section 2.22. In total, 224 proteins were identified between the isolates with a false discovery rate (FDR) of 0 %. However, many of these matches were cytosolic proteins arising from 1-2 peptide matches, indicating that there had been some carry over of cytosolic proteins into the OMP fraction. 164 of 224 identifications were shared between two isolates, while 19 proteins were identified only in 1A and 41 proteins identified only in 1B (Fig 4.4).

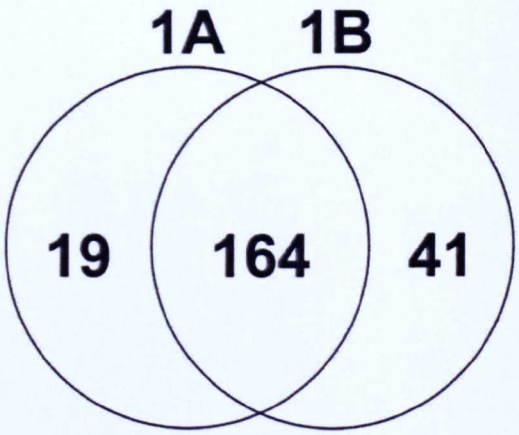

Figure 4.4. Venn diagram displaying number of protein identifications shared between isolates 1A and 1B, or detected only in carbapenem-susceptible isolate 1A, or carbapenem-resistant isolate 1B; generated by Scaffold software.

To make sure that the analysis was focused on outer membrane proteins, the lists of proteins identified in just one isolate were submitted to PSORTb (http://www.psort.org/psortb/), to confirm their subcellular localisation. Any proteins that were predicted to be cytosolic were excluded from further analysis. Similarly, any protein that could not be localised to a specific area of the cell or returned as hypothetical by BLASTp analysis, were also removed, to ensure that only the expression of membrane proteins was compared between isolates 1A and 1B. This left 14 proteins

identified in only 1A and 12 proteins identified in only 1B. While many cytosolic proteins were identified in the OMP fraction, the fractionation was still successful in enriching for more membrane proteins than in previous GeLC experiments. This resulted in more peptide identifications associated with outer membrane proteins, giving increased confidence to the identifications *e.g.* OmpK35 matched 16 peptides while TalB (cytosolic) only matched 2 peptides.

4.3.1 Proteins identified with roles in antibiotic resistance

There were five of the identified proteins thought to be involved in antibiotic resistance, including two identified in isolate 1A, including OmpK35 (protein 2; Table 4.1), one of the major porins of *K. pneumoniae*. When expression of OmpK35 is reduced or repressed, the OM permeability is lowered causing reduced accumulation of antibiotics (Martínez-Martínez, 2008). There was also an outer membrane porin protein C identified, which was identified in both isolates and returned as OmpK36 by BLASTp analysis (E = 0). However, it was only identified by 1 peptide in isolate 1B, below the set cut-off of 2 unique peptides. It is likely that the peptide was an artefact from another protein, as many more peptides (23) hit this protein in 1A. OmpK36 is also involved in antibiotic resistance, for the same reasons as OmpK35.

There were three proteins identified as present in isolate 1B: oligogalacturonate-specific porin protein KdgM (Protein 1; Table 4.2), which is likely to be a replacement porin for OmpK35 and OmpK36. KdgM is also known as OmpK26 and is thought to be essential for carbapenem-resistant isolates lacking OmpK35/36 (García-Sureda *et al.* 2011).

The *emrA* gene product (Protein 7; Table 4.2) is a periplasmic membrane-fusion protein (MFP) of the multidrug efflux pump EmrAB-TolC in *E. coli,* although its contribution to antibiotic resistance is masked by AcrAB-TolC (Tikhonova *et al.* 2009).

Protein 11 (Table 4.2), putative APH(3") streptomycin phosphotransferase or StrA, is an aminoglycoside phosphotransferase enzyme which confers resistance only against streptomycin (Ramirez & Tolmasky, 2011). With the loss of porins OmpK35 and OmpK36, and with the expression of these additional proteins, 1B seems the more resistant isolate based on the OMP profile.

Protein	Protein Identification	GI number	No. unique peptides
1	Conjugal transfer surface exclusion protein TraT	gi|152973591	7
2	Outer membrane protein 1A/OmpK35 porin	gi|238893985	16
3	Outer membrane protein for export and assembly of type 1 fimbriae	gi|238896402	15
4	Ferrichrome outer membrane transporter	gi|238893148	15
5	Periplasmic chaperone	gi|152971808	7
6	Klebicin B	gi|11342751	10
7	Type 1 major fimbrial subunit precursor	gi|238896399	2
8	Putative enzyme	gi|152972071	5
9	Maltose transporter subunit	gi|388479983	5
10	Protein FimF	gi|330011060	3
11	Hypothetical protein KP1_3289	gi|238895238	3
12	Malate dehydrogenase	gi|388479228	4
13	Lipid hydroperoxide peroxidase	gi|388477405	2
14	Dipeptide transport protein	gi|152972407	.3

Table 4.1. All the proteins identified as only expressed in isolate 1A and confirmed as membrane proteins by PSORTb. Any proteins which were confirmed as cytosolic by PSORTb were removed from further analysis.

4.3.2 Proteins involved in virulence/pathogenicity

As the OM proteome was being analysed, it was expected that many of the proteins identified might have a role in pathogenicity or virulence. As these are the proteins most likely in contact with both the natural and host environments and potentially with the immune system of the latter. There were eight proteins identified in 1A which were thought to be involved in virulence, these included the conjugal transfer surface exclusion protein TraT (Protein 1; Table 4.1). TraT is an OM lipoprotein that is usually encoded by conjugative plasmids and functions to prevent the transfer of its plasmid to bacteria that already carry that plasmid, or a very similar plasmid, thereby promoting the spread of its plasmid into diverse hosts (Sukupolvi & Connor, 1990). TraT also acts as a virulence factor by increasing the survival rate of host cells in serum and acts as a transporter across the outer membrane (Tomazella *et al.* 2011).

Four proteins coding for the assembly of fimbriae were identified only in 1A, they included an OMP for export and assembly of type 1 fimbriae (Protein 3; Table 4.1), which returned as FimD via BLASTp analysis (E = 0). FimD is an usher protein, involved in the polymerisation and translocation of the fimbrial proteins to the bacterial surface (Palomino *et al.* 2011). The periplasmic chaperone (Protein 5; Table 4.1), which returned as the fimbrial chaperone FimC via BLASTp analysis ($E = 2e^{-165}$), complexes with the structural subunits and initiates translocation via FimD (Gossert *et al.* 2008). Type 1 major fimbrial subunit precursor (Protein 7; Table 4.1) returned as FimA by BLASTp analysis ($E = 2e^{-122}$) and is the main structural subunit of the pilus (Puorger *et al.* 2011). Lastly is the adaptor protein FimF (Protein 10; Table 4.1), which links the adhesive tip of the pilus to the filamentous body and is also involved in the regulation of pilus biogenesis (Gossert *et al.* 2008).

Two proteins involved in iron acquisition; ferrichrome outer membrane transporter (Protein 4; Table 4.1), which returned as TonB-dependent siderophore receptor FhuA via BLASTp analysis (E = 0) and involved in transporting iron bound to siderophores across the membrane. There was also a dipeptide transport protein (Protein 14; Table 4.1), which returned as DppA (dipeptide permease) by BLASTp analysis. It is involved in the transport of dipeptides across the membrane and is also required for utilisation of haem as an iron source (Letoffe *et al.* 2006).

Protein	Protein Identification	GI number	No. unique peptides
1	Oligogalacturonate-specific porin protein KdgM	gi|330011167	17
2	Putative oligogalacturonide ABC transport system periplasmic binding component	gi|238896367	8
3	Putative oligogalacturonide ABC transport system ATP-binding component	gi|238896366	8
4	Anaerobic dimethyl sulfoxide (DMSO) reductase subunit A	gi|152969483	5
5	*nupC* gene product	gi|206578011	2
6	*secA* gene product	gi|206580132	4
7	*emrA* gene product	gi|206578040	3
8	Apolipoprotein N-acyltransferase	gi|152969253	3
9	Protease 4	gi|152969758	2
10	*fdoG* gene product	gi|206578515	4
11	Putative APH(3") streptomycin phosphotransferase	gi|152973750	2
12	GTP-binding protein	gi|238892284	2

Table 4.2. All the proteins identified as only expressed in isolate 1B and confirmed as membrane proteins by PSORTb. Any proteins which were confirmed as cytosolic by PSORTb were removed from further analysis.

Lipid hydroperoxide peroxidise (Protein 13; Table 4.1) returned as Tpx, a thiol peroxidise required for oxidative defence and also shown to be important for biofilm production in shiga-toxin producing *E. coli* (Kim *et al.* 2006).

There were two proteins identified in 1B that could potentially be involved in virulence, including: anaerobic dimethyl sulfoxide (DMSO) reductase subunit A (DmsA) (Protein 4; Table 4.2), which is able to utilise alternative electron acceptors for anaerobic growth and is said to contribute to virulence under anaerobic conditions in *Actinobacillus pleuropneumoniae* (Baltes *et al.* 2003).

GTP-binding protein (Protein 12; Table 4.2) returned as TypA/BipA via BLASTp analysis (E = 0), it is a translational GTPase which regulates virulence mechanisms in *E. coli*, possibly through control of protein translation (Margus *et al.* 2007). *Salmonella typhimurium* TypA mutants have shown reduced growth at lower temperatures, display reduced motility and have lower survival rates in murine macrophages (Sabbagh *et al.* 2012).

4.3.3 Proteins identified with other functions

There were other proteins identified that were considered unlikely to be involved in either antibiotic resistance or virulence, these included five proteins identified in 1A; Klebicin B (Protein 6; Table 4.1) is a colicin-type peptide molecule. It functions as a nonspecific endonuclease and is potentially used as a toxin against bacterial competitors (Riley *et al.* 2001).

Protein 8 (Table 4.1), putative enzyme returned as lipoprotein LppC by BLASTp analysis (E = 0), which has been described as a potential secreted virulence factor in *Actinobacillus pleuropneumoniae* (Zijnge *et al.* 2012), however, little is known about its function in *K. pneumoniae*.

Maltose transporter subunit (Protein 9; Table 4.1) returned as MalE via BLASTp analysis (E = 0), it seems likely that MalE is repressed by 1B to further reduce its membrane permeability.

Hypothetical protein KP1_3289 (Protein 11; Table 4.1) returned as YidY by BLASTp analysis ($E = 1e^{-163}$), an acid-inducible OMP (Stancik *et al.* 2002).

Malate dehydrogenase (Protein 12; Table 4.1) is involved in the TCA cycle for carbon utilisation.

There were also seven proteins identified with other functions in 1B, such as three proteins identified as belonging to an oligosaccharide transport system. These include oligogalacturonate-specific porin protein KdgM (Protein 1; Table 4.2). Protein 2 (Table 4.2), putative oligogalacturonide ABC transport system periplasmic binding component, returned as TogB by BLASTp analysis (E = 0). TogB is part of a multicomponent transporter which recognises the oligosaccharide substrates and translocates them across the membrane (Abbott & Boraston, 2008). Protein 3 (Table 4.2), putative oligogalacturonide ABC transport system ATP-binding component, returned as TogA by BLASTp analysis (E = 0). TogA is the cytoplasmic domain that utilises ATP for energy required for translocation (Abbott & Boraston, 2008). As OmpK26 has a potential role in antibiotic resistance, this complex may also have as yet unknown roles in resistance.

The *secA* gene product (Protein 5; Tale 4.2) is an OMP which works with the SecYEG translocase system to export partially folded proteins across the cytoplasmic membrane. SecA has ATPase activity, thus providing energy for protein translocation (Plessis *et al.* 2011 and Sardis & Economou 2010).

Protein 8 (Table 4.2), apolipoprotein N-acyltransferase, returned as Lnt via BLASTp analysis (E = 0). It is an essential protein in *E. coli* and catalyses the last step of lipoprotein modification before translocation to the outer membrane (Narita & Tokuda, 2011).

Protein 9 (Table 4.2), protease 4, returned as signal peptide peptidase SppA via BLASTp analysis (E = 0). SppA is a serine protease which cleaves the signal peptide from lipoproteins, allowing mature lipoproteins to insert into the membrane (Wang *et al.* 2009).

Protein 10, (Table 4.2), the *fdoG* gene product is the major subunit of formate dehydrogenase-O, which works to reduce formate under aerobic conditions. There is a similar complex which acts under anaerobic conditions and it is thought that having both complexes allows rapid switching of metabolic pathways in response to changes in environmental oxygen (Benoit *et al.* 1998).

The *nupC* gene product (Protein 5; Table 4.2) transports pyrimidine nucleosides (but not purines) across the membrane and into the cell (Patching *et al.* 2005).

4.4 Chapter summary

The aims of this study were to characterise changes in the *K. pneumoniae* OM proteome potentially caused by differential porin expression and to investigate whether any other OMP expression was affected, which could act as potential markers of this carbapenem resistance mechanism. The technique of 1D SDS-PAGE to visualise the OMP composition of suspected non-carbapenemase-mediated carbapenem-resistant isolates has been described previously (Doumith *et al.* 2009; Martínez-Martínez, 2008) and here it was successful in confirming the suspected resistant mechanism in this clinical pair of isolates (Webster *et al.* 2010). However, very little information on the proteins themselves was available in the literature, therefore the OMP profiles of the isolates were analysed by LC-MS/MS to obtain identifications for all the proteins present in the OMP fraction. The bottom-up proteomics approach used here yielded a greater amount of information about the isolates and the proteome changes as a result of carbapenem resistance, including many changes in addition to the loss of OmpK35/36 porins.

Few studies have investigated the *K. pneumoniae* proteome, especially the OM proteome. For example, Kurupati *et al.* analysed the immunogenicity of the proteins in an OMP fraction of *K. pneumoniae* (Kurupati *et al.* 2006). While Cho *et al.* analysed the OMPs of imipenem-resistant *K. pneumoniae,* they were measuring the effects of a green tea extract on the OM proteome rather than changes caused by imipenem resistance (Cho *et al.* 2011). To our knowledge, no work has been published investigating changes in the OM proteome with respect to investigating carbapenem resistance in *K. pneumoniae.*

There were five proteins thought to have roles in antibiotic resistance identified in the OMP analysis, such as the OmpK35/36 porins, which need to be repressed to give the carbapenem-resistant phenotype and neither were expressed in 1B. The little-known porin OmpK26 was also expressed in 1B, this porin is known to be expressed in carbapenem resistant isolates lacking

OmpK35/36 expression (García-Sureda *et al.* 2011). Although many more OMP profiles need to be tested to confirm this observation, the presence of OmpK26 could be a marker protein for this carbapenem-resistant phenotype.

The streptomycin resistance protein APH(3'') and efflux protein EmrA were also expressed only in isolate 1B. It may be possible that APH(3'') was from a plasmid acquired by 1B, however *emrA* is chromosomally located, indicating that this carbapenem-resistance mechanism could be involved with the induced expression of drug efflux pumps. This finding suggests that low-level efflux activity may be playing a role in carbapenem non-susceptibility in this isolate, although this requires further confirmation by testing other isolates.

1B was found to express SecA, required for the Sec protein transport system, which delivers (among other proteins) β-lactamases such as TEM, AmpC and CTX-M enzymes to the periplasm (Pradel *et al.* 2009). Therefore, SecA may be important for resistance against β-lactam antibiotics, particularly in combination with expression of EmrA and repression of ompK35/36. 1B has reduced expression of OmpK35/36 and expression of a CTX-M ESBL and is also expressing MDR efflux protein EmrA and OM transporter SecA. The collective activity of these proteins is likely to confer higher resistance to carbapenems than just OmpK35/36 loss through reduction of the periplasmic concentration of CTX-M.

There were many virulence factors expressed in both 1A and 1B respectively. Some seemingly important proteins for virulence were missing from 1B, including FhuA and DppA (iron acquisition proteins). Isolate 1B also lacks the machinery for the synthesis and assembly of fimbriae or pili, which are one of the main virulence-associated properties of *K. pneumoniae,* required for attachment to mammalian cells to initiate colonisation and infection. FimD is an usher protein, essential for the polymerisation and translocation of the fimbrial proteins to the bacterial surface. Fimbrial ushers are among the largest pores in the OM (Palomino *et al.* 2011), which could explain why 1B does not express any of the proteins due to the size of FimD porin being used for antibiotic entry. Perhaps the lack of these proteins could contribute to reduced pathogenicity in an infection, alternatively, isolate 1B may have lost the proteins as an immunoevasion strategy (to become 'invisible' to the immune system) and would be valuable to investigate further.

Klebicin B, a colicin-type protein was also absent form the 1B OMP fraction. Assuming it is plasmid encoded, 1B may not be expressing the plasmid, or may even have lost it. This could also contribute to it being outcompeted by other bacteria. 1B also lacked iron acquisition proteins FhuA and DppA (required for haem uptake) and potential virulence factor TraT. With all these proteins absent, it is possible that 1B may not be as able to colonise a host and initiate infection as well as 1A.

Overall, the results suggest that 1B lost or reduced the expression of many membrane transport proteins, as reduced permeability is what confers carbapenem non-susceptibility. Therefore, it is logical for 1B to restrict as many entrances to the cell as is feasible. This is why OmpK35 and OmpK36 were not expressed (which was expected), but possibly TraT, MalE and FimD as well. 1B has also expressed other membrane transport proteins to replace those that were repressed *e.g.* the Tog system, OmpK26 used by Tog system and the transporter NupC. There were also many other proteins with other functions identified in both isolates but their relevance to the resistance mechanism has yet to be determined.

As 1B shows such an altered virulence/resistance phenotype compared with 1A, it may be possible that some genetic reorganisation had taken place *e.g.* regions of gene deletions, insertions etc. Therefore, to validate the results generated by this OMP GeLC technique, the genomes of the isolates would ideally be sequenced to determine their genetic similarity. The proteomics techniques used in this study have detected the suspected loss of OmpK35/36 as well as additional changes to the OMP profiles between this clinical pair of isolates, which were potentially associated with the acquisition of a carbapenem-resistant phenotype. The expression differences detected by proteomics could be result of the loss/acquisition of genetic material. If this is confirmed by DNA sequence analysis it could give an insight into the selection process bacteria undergo while colonising the host and acquiring resistance. If the changes observed are purely protein expression differences and not underlined by genetic changes, then they are indicative of major changes in regulatory networks affecting porins, iron uptake and many other functions and could not have been elucidated with traditional phenotypic and genetic amplification or sequencing assays. This study also revealed changes in expressed proteins that could have implications on the

antibiotic resistance and pathogenic capabilities of organisms which acquire a similar resistance mechanism.

5. Results
Tigecycline resistance in *Acinetobacter baumannii*

5.1 Introduction of isolates

Tigecycline is an agent of last resort to tackle multidrug resistant bacteria. *A. baumannii* is known for its pandrug resistance potential (Falagas & Bliziotis, 2007), including the ability to develop efflux-mediated resistance to tigecycline; it is critical to investigate strategies for overcoming efflux-mediated resistance. The protein expression profile of *A. baumannii* has been characterised previously (Soares *et al.* 2010; Fernandez-Reyes *et al.* 2009 and Shin *et al.* 2009), as with the mechanism of tigecycline resistance (Ruzin *et al.* 2007; Hornsey *et al.* 2010a). However, the potential of a proteomic approach to further investigate this resistance mechanism has not yet been realised.

The isolates analysed in this study included: a clinical pair of *A. baumannii,* recovered before (AB210; tigecycline MIC of 0.5 mg/L) and after (AB211; tigecycline MIC of 16 mg/L) tigecycline therapy, a laboratory-mutant derived from AB210 (AB210-6; tigecycline MIC of 64 mg/L) and a knockout-mutant derived from AB211 (AB211Δ*adeB*; tigecycline MIC of 0.5 mg/L) to give a group of extremely closely related isolates. Comparative genomics of this clinical pair demonstrated a high sequence similarity between these organisms (Hornsey *et al.* 2010a), making these isolates a highly desirable candidate group for comparative proteomics. The fact that the isolates are closely related should minimise protein expression differences related to strain heterogeneity often observed in proteomic investigations. Thus allowing the detection of expression differences directly linked to tigecycline resistance.

DIGE was chosen as the method for quantification of protein expressions as 2DGE techniques had been optimised previously (see methods section 2.12) and the isolates were all highly similar. As there were unlikely to be major differences in the proteome content, the aim was to use DIGE to highlight the subtle changes in protein expression/abundance to reveal new insights into efflux-mediated resistance mechanisms.

The aim of this study was to identify expression changes in proteins potentially associated with the efflux-mediated tigecycline resistance mechanism, thereby characterising which proteins may be required for upregulation of the efflux pump. These proteins could potentially provide

novel drug targets to inhibit this resistance mechanism and restore susceptibility to a range of antimicrobial agents.

5.2 Protein profiling of the extracts by 2-D gel electrophoresis

5.2.1 Separation on gradient of pH 4-7

The protein extracts from *A. baumannii* were obtained using protocols previously optimised on *E. coli* (see methods sections 2.5.1 and 2.5.2). The crude protein extracts from each replicate of every isolate were separated on 2-D gels prior to CyDye labelling (Figures 5.1-5.4). These initial separations were to demonstrate that (i) the extracts were free from any charged or insoluble contaminants that could cause streaking and (ii) the proteins would separate with good resolution using the specified pH gradient (see Figures 5.1-5.4). The gels shown here yielded the highest number of resolved spots and were subsequently used as picking gels to supply the material needed for protein identification. As the spot-picking robot could not image CyDye-labelled proteins, SYPRO-stained gels were used for spot picking and subsequent protein identification.

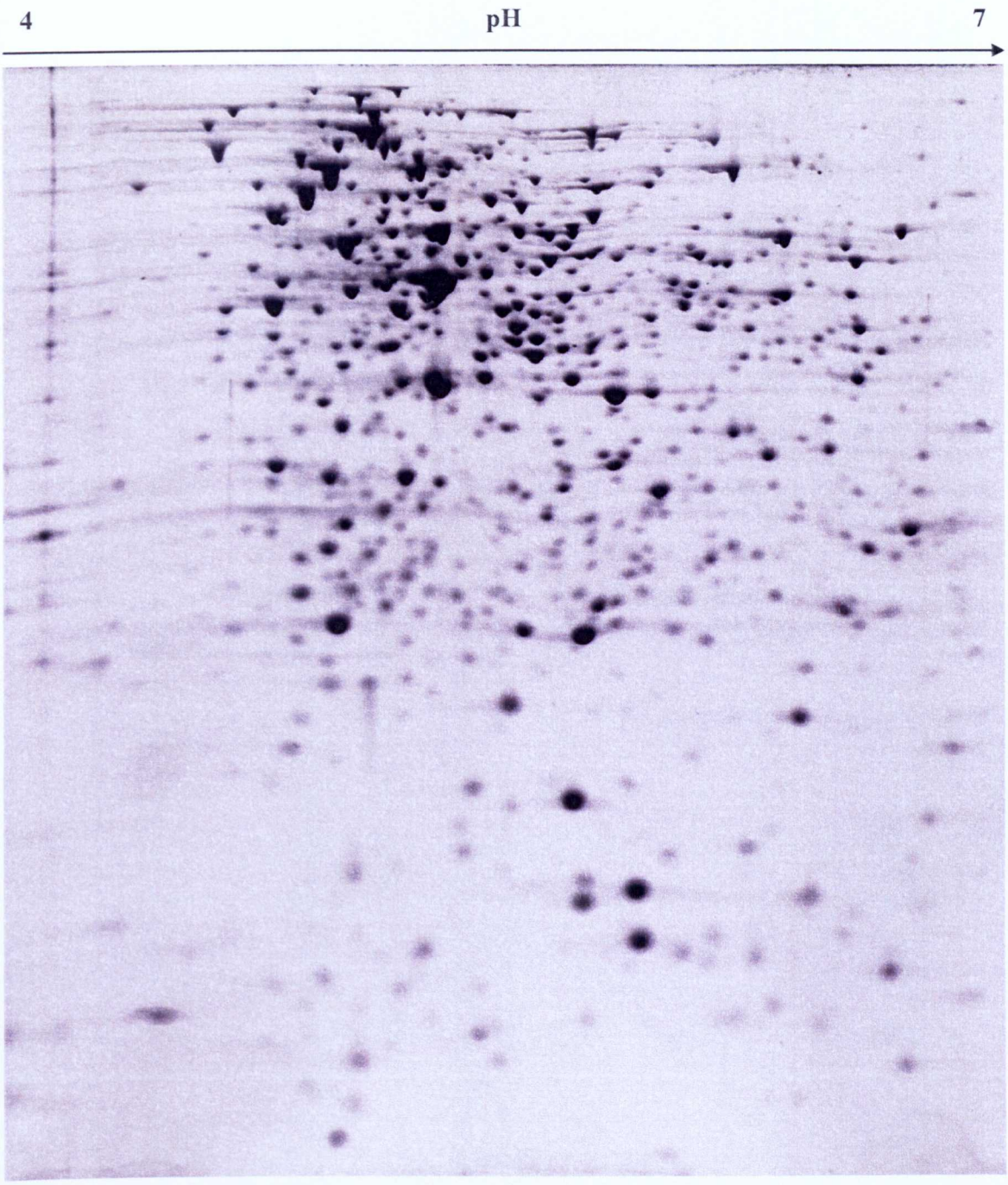

Figure 5.1 2DGE profile of tigecycline-susceptible clinical isolate AB210. Total cell extract was separated over a gradient of pH 4-7 and a 12% polyacrylamide gel. The proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

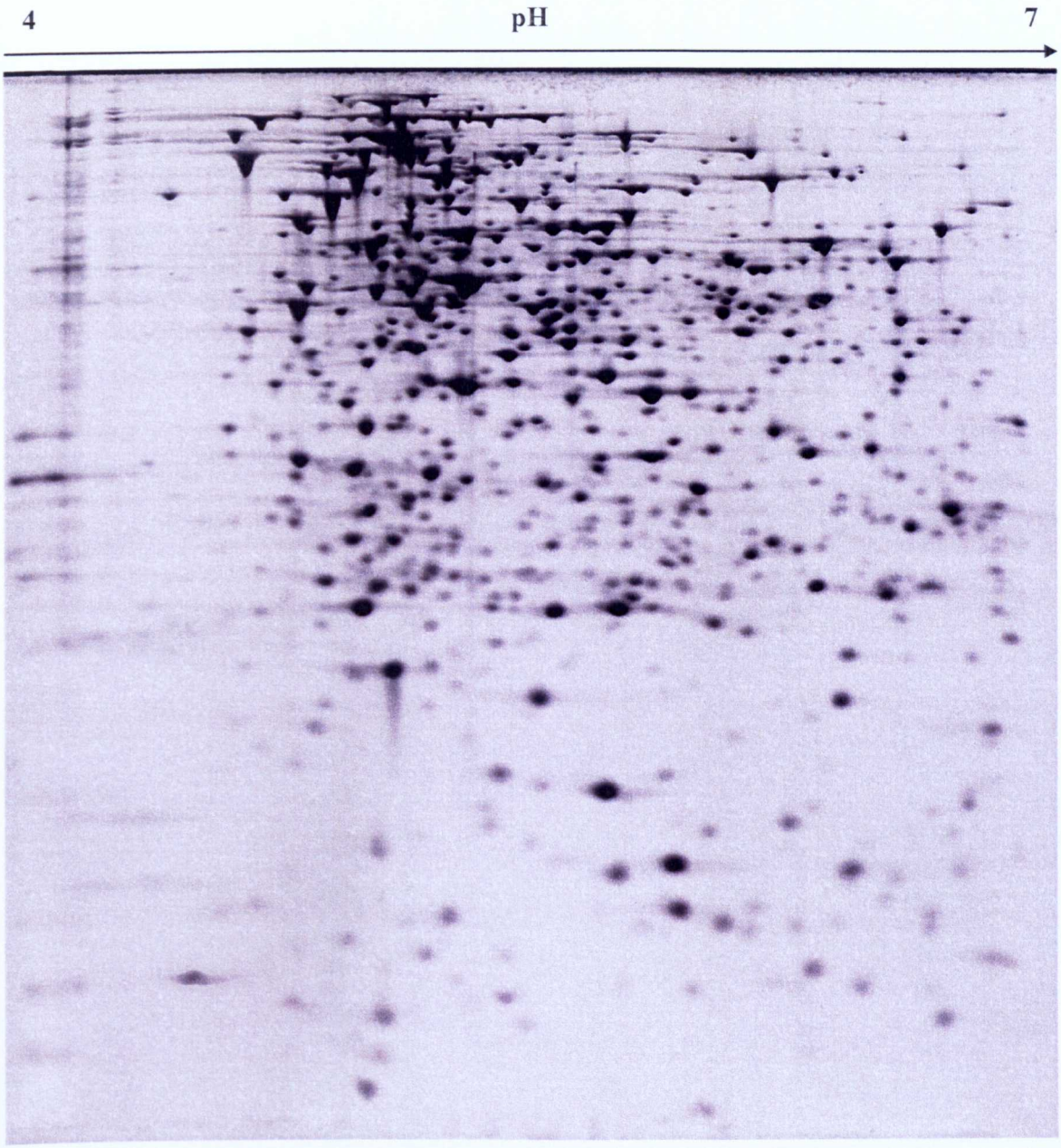

Figure 5.2 2DGE profile of tigecycline-resistant laboratory mutant AB210-6. Total cell extract was separated over a gradient of pH 4-7 and through a 12% polyacrylamide gel. The proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

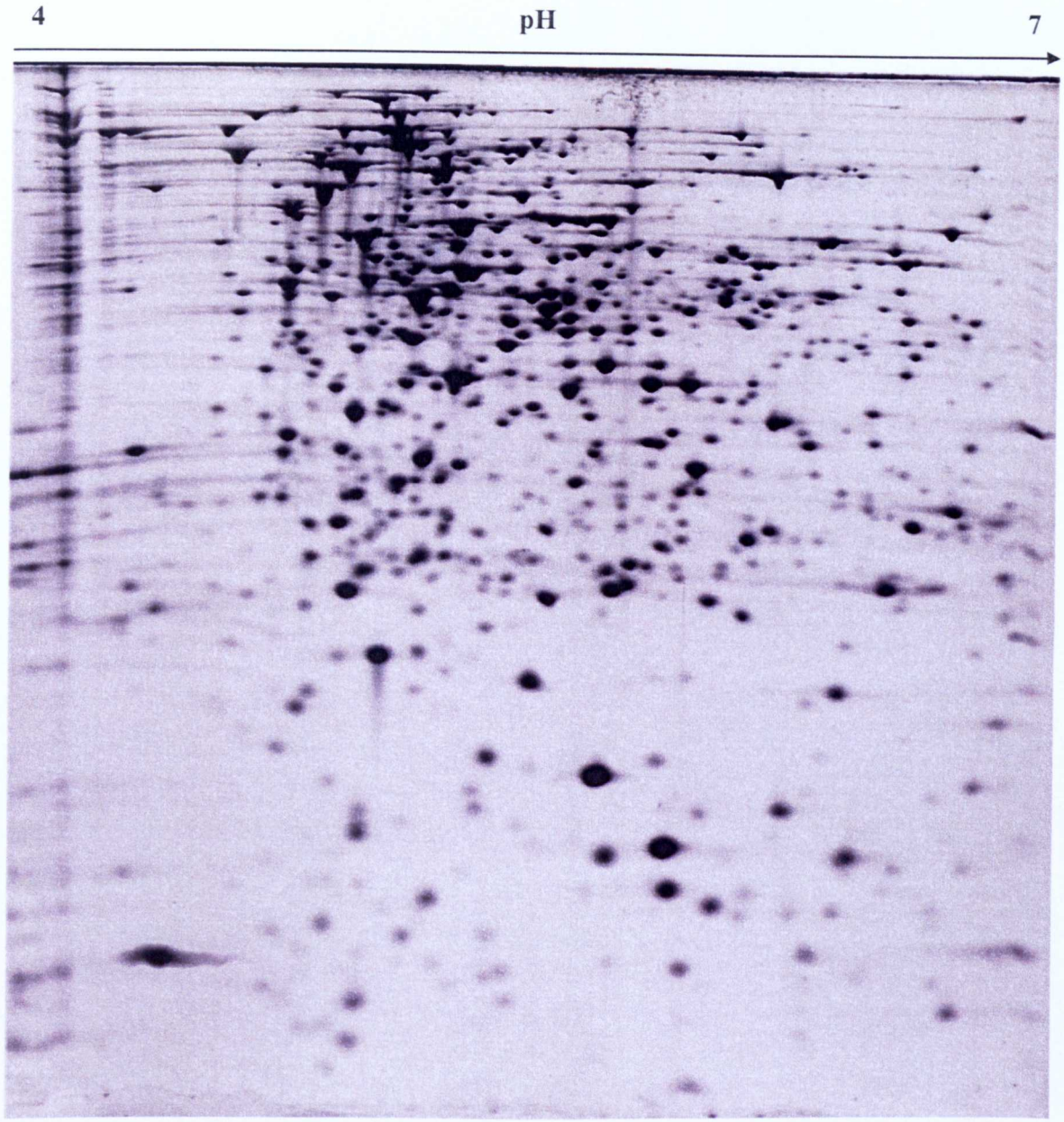

Figure 5.3 2DGE profile of tigecycline-resistant clinical isolate AB211. Total cell extract was separated over a gradient of pH 4-7 and a 12% polyacrylamide gel. The proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

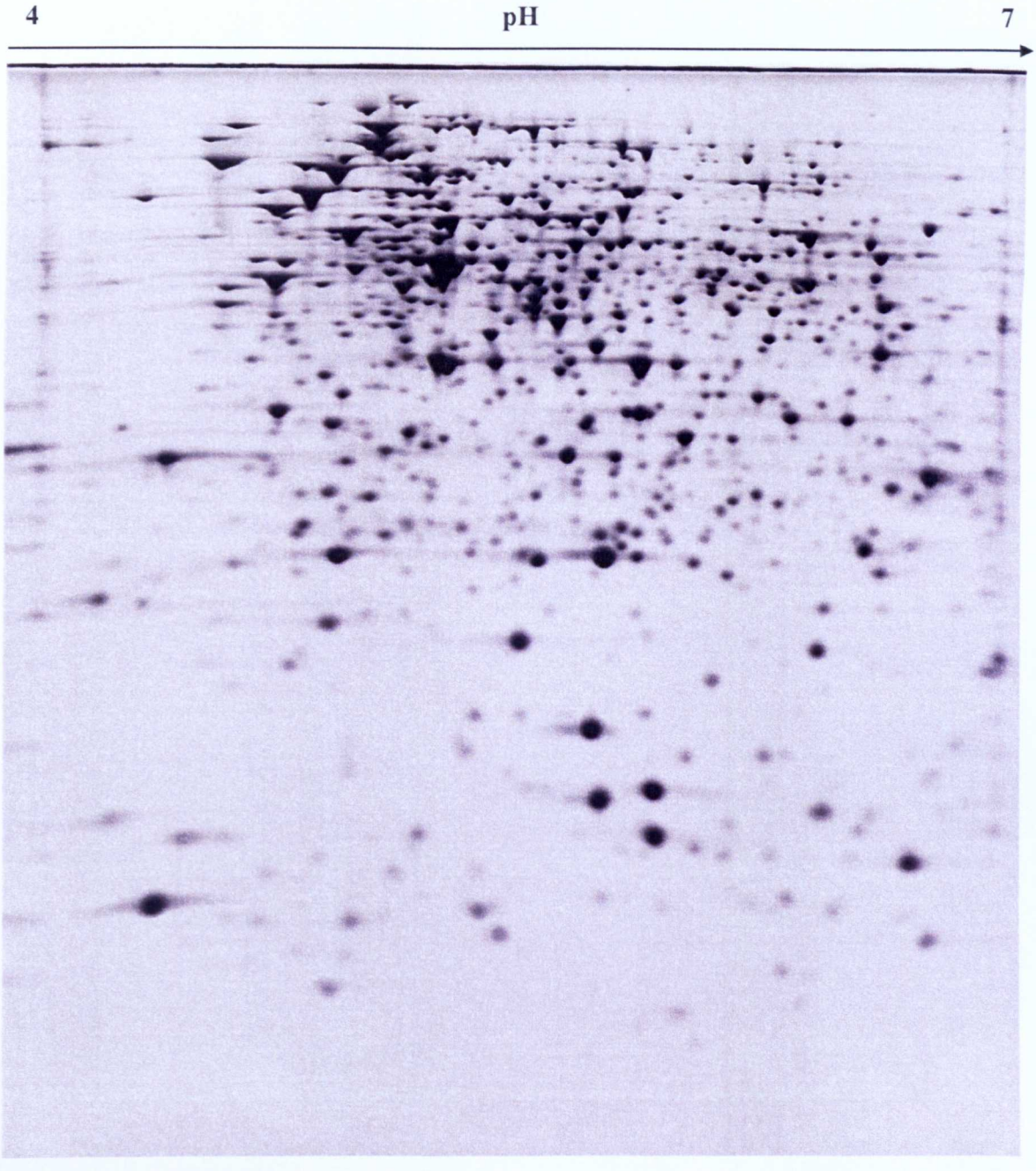

Figure 5.4 2DGE profile of tigecycline-susceptible knockout mutant AB211Δ*adeB*. Total cell extract was separated over a gradient of pH 4-7 and a 12% polyacrylamide gel. The proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

Once all extracts had been optimised, a DIGE experiment was designed to label all three replicates of each extract with alternating dyes (see methods section 2.10) as in table 5.1 below.

Gel no.	Labelled with Cy3	Labelled with Cy5
1	AB210 (1)	AB211 (3)
2	AB210-6 (3)	AB210 (2)
3	AB210 (3)	AB211Δ*adeB* (1)
4	AB211 (2)	AB210-6 (1)
5	AB211Δ*adeB* (2)	AB211 (1)
6	AB210-6 (2)	AB211Δ*adeB* (3)

Table 5.1 DIGE experimental setup for *A. baumannii* protein extracts with the biological replicate number in brackets. An internal standard was also included in each gel, this was composed of an equal amount of each sample and labelled with Cy2 for more accurate spot analysis.

5.3 Separation of DIGE labelled proteins over a gradient of pH 3-10

The DIGE procedure (see methods section 2.10) was first attempted on IPG gradients of pH 3-10 (Fig. 5.5), with the aim of resolving efflux pump proteins as well as cytosolic proteins (as the AdeAB proteins have high pI values c. pH 9). However, the gradient of pH 3-10 proved to be unsuitable as the majority of spots were poorly resolved. As a result many proteins may not have been visualised due to masking by proteins of greatest abundance, so a pH 4-7 gradient was investigated, although using this narrower gradient would mean the AdeAB proteins would not be seen on the gels, it was determined that the increased number of resolved spots would provide more information regarding tigecycline resistance.

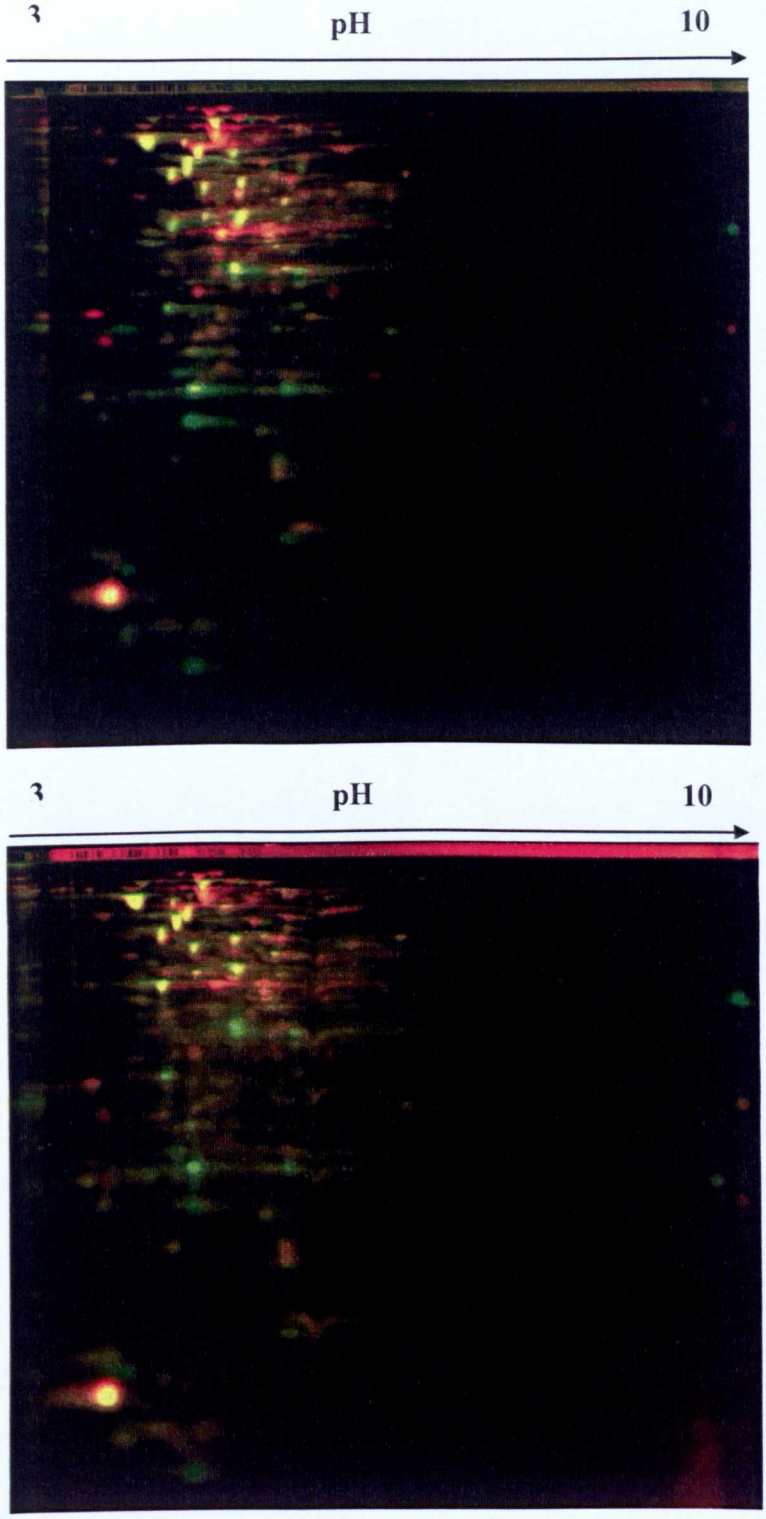

Figure 5.5 Examples of *A. baumannii* protein extracts separated on 2-D DIGE gels using gradients of pH 3-10 and a 12% polyacrylamide gel. The separated proteins were labelled with CyDye minimal dyes and visualised using an Ettan Dalt imager (GE Healthcare).

The DIGE experiment was repeated on pH 4-7 gradients, yielding good resolution and separation with changes in expression that were statistically significant. When performing DIGE, all the protein extracts were labelled and separated together in one large experiment, whereby three replicates of four extracts were run, at two samples per gel, requiring six gels. The SameSpots software (version 3.3.2) was able to separate each image into its respective dyes, providing three images of each gel taken at different wavelengths. SameSpots then allowed the user to select which images to use for comparison. From the example experiment outlined in table 5.1, it was determined that the most applicable comparisons would be: i) the clinical pair AB210 *vs.* AB211 ii) the tigecycline-susceptible AB210 *vs.* tigecycline-resistant lab mutant AB210-6 iii) both tigecycline-resistant isolates AB211 and AB210-6 and iv) AB211 *vs.* the tigecycline-susceptible knockout mutant AB211Δ*adeB*.

5.4 DIGE comparison of the pre-therapy (AB210) and post-therapy (AB211) clinical isolates

Hornsey *et al.* (2010) showed that expression of the *adeAB* operon was up-regulated in tigecycline-resistant isolate AB211 versus AB210 (Hornsey *et al.* 2010a). However, components of the AdeABC efflux pump were predicted *in silico* not to separate sufficiently on pH 4-7 gels, therefore, the protein extracts were also separated and analysed on pH 6-11 gels. However, no differential protein expression was observed in this pH region for Ade proteins. The limitations of the 2D electrophoresis system could explain this, as high pI and membrane proteins are poorly represented on polyacrylamide gels and should be analysed using a gel-free system where possible. Nonetheless, gel-based DIGE was chosen to explore broad-scale protein expression differences of the isolates and not merely the membrane proteins.

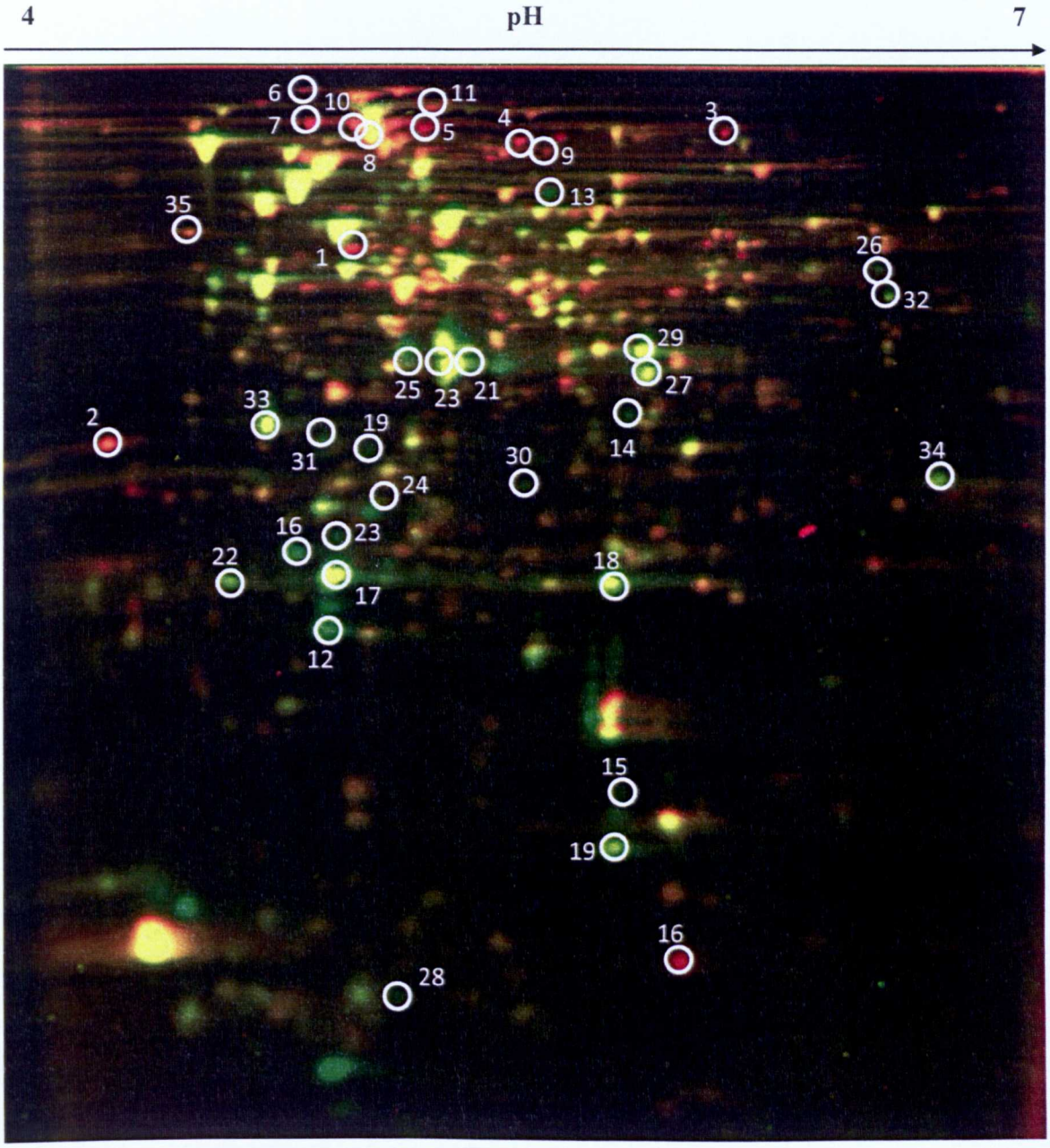

Figure 5.6 2-D separation of DIGE-labelled proteins using extracts from AB210 (green) and AB211 (red) separated over a pH gradient of 4-7 and through a 12% polyacrylamide gel. Numbers correspond to the proteins in tables 5.3 and 5.4.

In total c. 650 protein spots were detected by SameSpots software (version 3.3) over a pH range of 4-7, eight protein spots were detected only in the tigecycline-susceptible isolate AB210 and five only in the tigecycline-resistant isolate AB211 (Table 5.2). A further 35 proteins were identified in both isolates but displayed differential expression (Figure 5.6); 24 proteins showed increased expression (≥2-fold) in AB210 (Table 5.3) and 11 proteins showed increased expression in AB211 (Table 5.4). Several of these differences may be associated with bacterial virulence and were clustered into the following functional groups: (i) antibiotic resistance-related proteins; (ii) attachment/biofilm formation-related proteins; and (iii) iron acquisition-related proteins.

5.4.1 Proteins detected only in one isolate

The proteins detected by DIGE in one isolate included a putative lactam utilisation protein (spot 1; Table 5.2, Fig. 5.7), which was detected only in the tigecycline-susceptible isolate AB210. A BLASTp search showed high similarity with proteins belonging to the LamB/YscF superfamily (E value = $8e^{-97}$), which includes the LamB carbohydrate porin, a specific maltose transporter. Spot 9 (Table 5.2, Fig. 5.8) was detected only in tigecycline-resistant isolate AB211 and was identified as Porin B, a carbohydrate-selective porin belonging to the OprB family. The appearance of this protein in AB211 suggests expression of an alternative transporter in response to the absence of the lactam-utilising LamB family protein.

The other proteins unique to AB210 were poorly characterised proteins lacking clearly defined cellular functions. These included a putative polysaccharide biosynthesis protein (spot 6; Table 5.2, Fig. 5.7), highly similar to *N*-acylneuraminate cytidylyltransferases, which synthesise *N*-acetylneuraminic acid polymers. This is an important virulence factor in pathogenic bacteria such as *Escherichia coli*, *Neisseria meningitidis*, *Haemophilus ducreyi* and group B streptococci (Mizanur & Pohl, 2008). There were four hypothetical proteins unique to AB210 (Table 5.2); spot 3 showed high similarity to phosphopantothenoylcysteine synthetase / carboxylase (E = 0) which is involved in the synthesis of coenzyme A; spot 4 was highly similar to ferridoxin; spot 7 was potentially a member of a Bacterial OB-fold (BOF) superfamily, which consists of sub-families with diverse functions including an enterotoxin family and DNA-binding domain family (Ginalski

et al. 2004) and spot 8 showed high similarity to a GcvT-like aminomethyltransferase ($E = 6e^{-172}$). GcvT is a glycine cleavage system-protein, working to convert glycine to serine when cellular concentrations are high. There was a single hypothetical protein that was unique to AB211 (spot 11; Table 5.2) but no conserved domains could be identified (as searched in InterProScan) and only matched to hypothetical proteins from the *Acinetobacter* genus by BLASTp analysis.

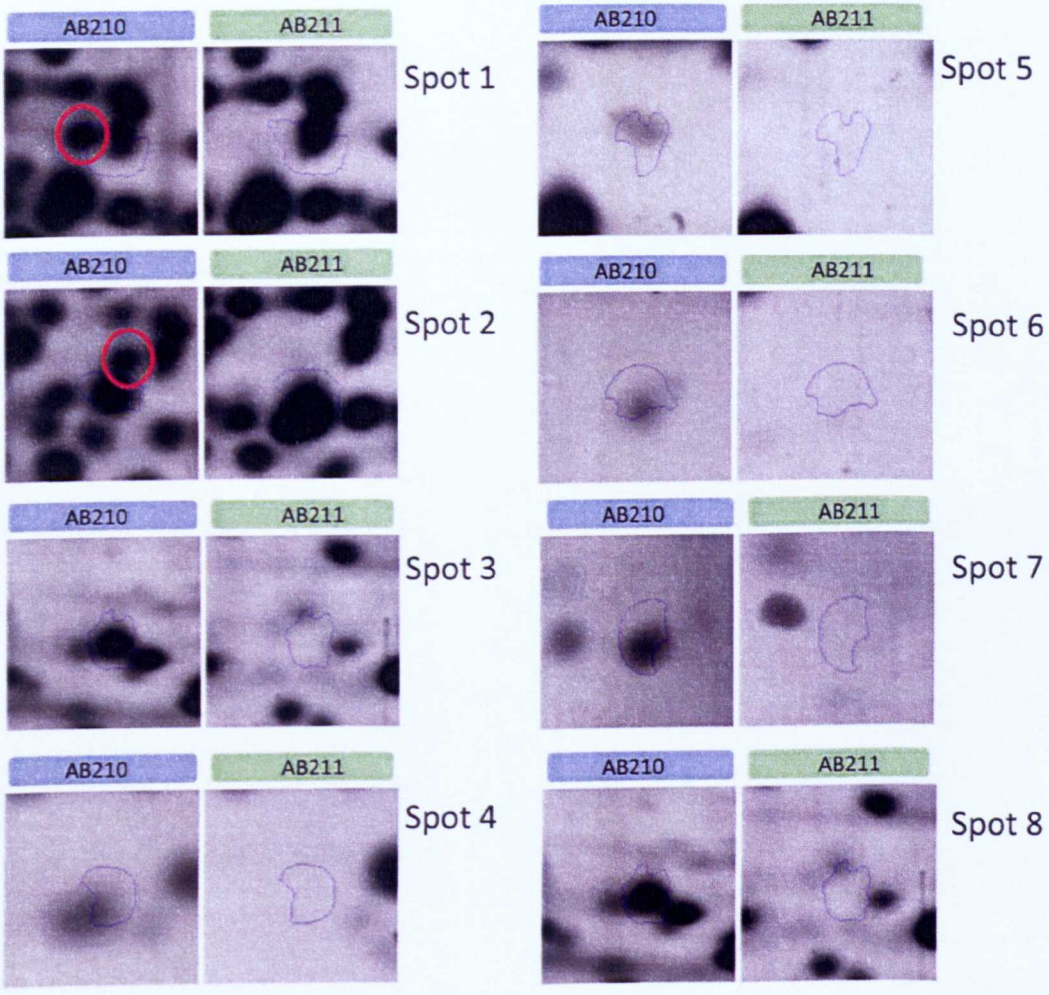

Figure 5.7 Proteins spots which were detected in AB210 and not in AB211 (see Table 5.2). Red circles highlight spots that were incorrectly missed by SameSpots software and manually corrected.

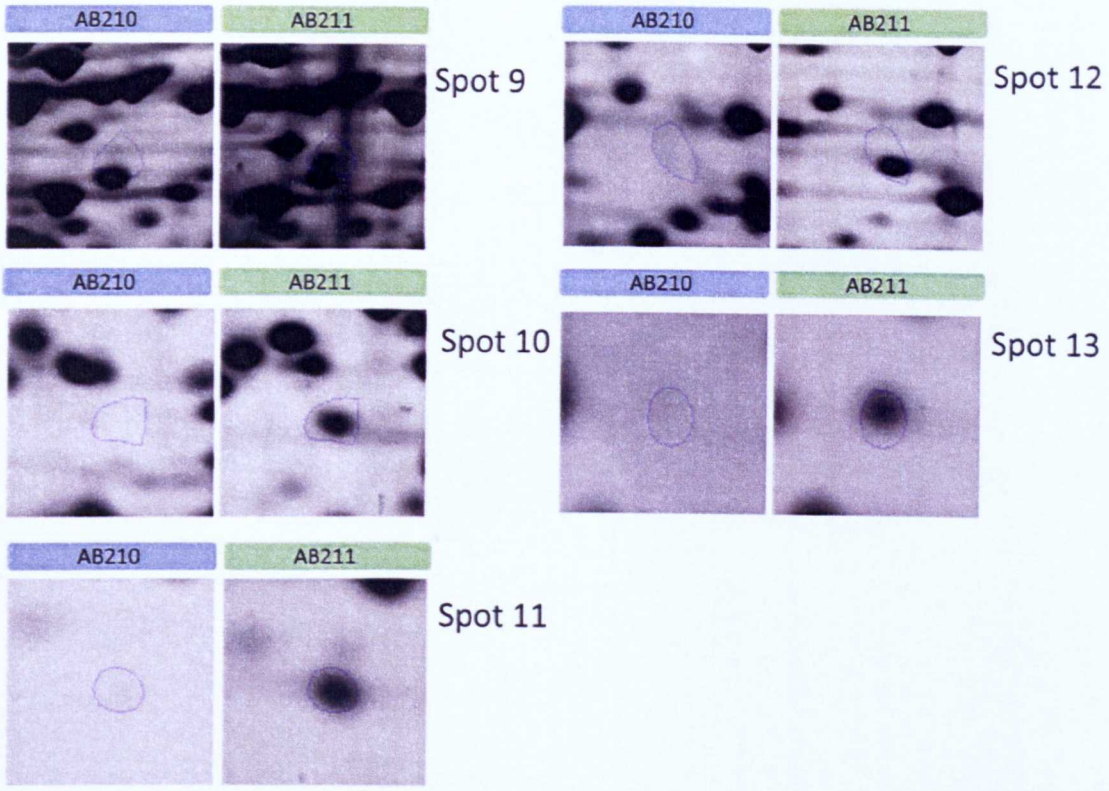

Figure 5.8 Protein spots which were detected in AB211 but not in AB210 (see Table 5.2)

	Protein Spot	Protein ID	GI number	Mol Wt.	No. unique peptides	Confirmed as unique in genome?
AB210	1	Putative lactam utilization protein *(A. baumannii* ATCC 17978)	gi|126641313	19 kDa	3	-
	2	Aminoglycoside 6' N-acetyl transferase type Ib *(A. baumannii* ACICU)	gi|184156543	24 kDa	4	Y
	3	Hypothetical protein A1S 2917 *(A. baumannii* ATCC 17978)	gi|126642940	40 kDa	6	-
	4	Hypothetical protein ABSDF1503 *(A. baumannii* SDF)	gi|169633191	14 kDa	4	-
	5	Putative NADPH quinone reductase modulator of drug activity B *(A. baumannii* ACICU)	gi|184157604	20 kDa	4	Y
	6	Putative polysaccharide biosynthesis protein *(A. baumannii* SDF)	gi|169632090	26 kDa	3	-
	7	Hypothetical protein ACICU 02431 *(A. baumannii* ACICU)	gi|184158751	13 kDa	2	-
	8	Hypothetical protein ABSDF0627 *(A. baumannii* SDF)	gi|169632509	27 kDa	2	-
AB211	9	Porin B *(A. baumannii* SDF)	gi|169633605	49 kDa	7	-
	10	Putative type 1 pili subunit CsuA/B protein *(A. baumannii* ACICU)	gi|184158737	19 kDa	4	-
	11	Hypothetical protein ACICU 01910 *(A. baumannii* ACICU)	gi|184158230	15 kDa	2	-
	12	Putative phospholipase A1 precursor PldA *(A. baumannii* ATCC 17978)	gi|126641964	40 kDa	10	-
	13	3 dehydroquinate dehydratase *(A. baumannii* ATCC 17978)	gi|126642054	15 kDa	4	-

Table 5.2 Identifications assigned to protein spots that were detected only in either AB210 or AB211. Two of these 'unique' assignments were confirmed by Hornsey *et al.* (2010a).

5.4.2 Changes in antibiotic resistance profile

Two of the eight proteins detected only in AB210 were associated with resistance to antibiotics or other compounds. These included the aminoglycoside-modifying enzyme, aminoglycoside 6' N-acetyl transferase type 1b or AAC(6')-Ib (spot 2; Table 5.2). This finding is consistent with the eight-fold reductions in aminoglycoside MICs for AB211 versus AB210 (see methods 2.2 for *A. baumannii* MICs) (Hornsey *et al.* 2010a).

The other resistance-associated protein, spot 5 (Table 5.2), was identified as a putative NADPH quinone reductase (modulator of drug activity B or MdaB). This protein has previously been described in *Escherichia coli* and grants protection to the cell from quinoid compounds. These occur naturally as electron carriers in the electron transport chain, but can cause toxicity through increased production of intracellular reactive oxygen species (Adams & Jia, 2006).

5.4.3 Iron acquisition proteins

Spot 13 (Table 5.2) was identified as 3-dehydroquinate dehydratase and appeared unique to AB211 by DIGE. This protein is associated with iron acquisition and is known as QuiB or AroD in the *Enterobacter*iaceae (Elsemore & Ornston, 1995), where it is part of the biosynthetic pathway of shikimate, a precursor for aromatic amino acid and also catechol-based siderophore production, implying that AroD could have an indirect role in iron acquisition.

Consistent with this, an outer membrane receptor for monomeric catechols, which can be used to sequester iron, showed an increase in expression of 3.1-fold in isolate AB211 (spot 4; Table 5.3). Using BLASTp analysis, this protein showed a high degree of similarity to TonB-dependent iron receptor protein BfrD (E value = 0).

Expression of a ferrichrome iron receptor protein (spot 9; Table 5.3) was also increased 2.1-fold in AB211. The increased expression of this protein and the BfrD catechol receptor protein, combined with the presence of AroD suggests that AB211 may be better equipped to sequester iron

Spot no.	Protein ID	GI number	Mol Wt.	No. unique peptides	Fold expression increase *vs.* AB210	*p* value ANOVA
1	Hypothetical protein ACICU_00960]	gi|184157280	53 kDa	15	4.9	**0.037**
2	Outer membrane protein	gi|260557941	32 kDa	13	3.9	**0.027**
3	P pilus assembly protein porin PapC	gi|184158735	93 kDa	22	3.2	**0.002**
4	Outer membrane receptor for monomeric catechols	gi|184156805	81 kDa	20	3.1	**0.035**
5	ATP dependent protease Hsp 100	gi|126641234	92 kDa	21	3	**0.025**
6	Methionine synthase I cobalamin binding subunit MetH	gi|184157253	136 kDa	18	2.8	**0.004**
7	Organic solvent tolerance protein OstA	gi|184157914	92 kDa	18	2.3	**0.006**
8	Outer membrane protein assembly complex YaeT protein	gi|215483173	93 kDa	27	2.2	**0.018**
9	Ferrichrome iron receptor protein	gi|184158352	77 kDa	11	2.1	**0.014**
10	Elongation factor G	gi|169634057	79 kDa	20	2	**0.032**
11	ATP dependent protease Hsp 100 part of multi chaperone system with DnaK DnaJ and GrpE	gi|169633163	95 kDa	25	2	**0.018**

Table 5.3 Proteins that were highlighted by SameSpots software as displaying increased expression in AB211 *vs.* AB210. The p-values displayed were calculated by SameSpots software and show the likelihood of observing this expression difference if no real difference existed *e.g.* a small p-value indicates a small chance of observing this expression difference if there was no real difference. GI number stands for gene identifier number, a standard naming system for proteins used in NCBI.

from its environment than AB210 and consequently, may be more virulent *in vivo* (Zimbler *et al.* 2009).

5.4.4 Changes in protein expression related to pilus production, attachment and biofilm formation

It has been reported that *A. baumannii* grown under iron-limited conditions demonstrated increased biofilm formation when compared with the same isolate in iron-rich conditions, suggesting that proteins involved in biofilm formation and iron acquisition may share common promoters/regulators (Tomaras *et al.* 2003 and Shin *et al.* 2009). Consistent with this, other proteins showing increased expression in AB211 were predicted to mediate attachment and biofilm formation, including the outer membrane usher protein PapC (spot 3; Table 5.3), which recruits pilus subunits, catalyses their assembly and translocates them across the outer membrane (Huang *et al.* 2009). PapC expression was increased 3.2-fold in isolate AB211 and was consistent with the presence of the CsuA/B subunit (spot 10; Table 5.2). This was detected only in AB211 and is a secreted pilus subunit required for motility and biofilm formation (Vashist *et al.* 2010 and Siroy *et al.* 2006). It was shown by Tomaras *et al.* that correct pilus assembly was essential for biofilm formation in *A. baumannii* (Tomaras *et al.* 2003)

PldA or phospholipase A1 (spot 12; Table 5.2) was also identified as unique to isolate AB211. It is involved in biogenesis and modification of the cell envelope and is a virulence factor known to promote colonisation of *Yersinia enterocolitica* (Istivan & Coloe, 2006).

The expression of outer membrane protein assembly complex YaeT was also increased 2.2-fold in isolate AB211 and is required for the insertion of proteins into the outer membrane, as well as for autotransporter secretion in certain organisms (Jain & Goldberg, 2007). The increase of YaeT (spot 8, Table 5.3) in isolate AB211 could be a consequence of the increased traffic of outer membrane proteins (Porin B, phospholipase A and the majority of proteins with increased expression in AB211), which require chaperoning to be correctly inserted into the membrane to function correctly.

Organic solvent tolerance protein OstA (spot 7, Table 5.3) showed increased expression in AB211 by 2.3-fold, it is also known as lipopolysaccharide (LPS) assembly protein or LptD. This protein is required for LPS biogenesis, specifically the transport of LPS across the outer membrane and assembly at the cell surface (Chng *et al.* 2010).

The RstA protein of the two-component regulatory system response regulator RstA/B (spot 31; Table 5.4), showed a 2.2-fold increase in expression in AB210 when compared with AB211. This protein is also known as BfmR and is required to repress transcription of a number of genes implicated in iron uptake, responding to stress conditions and attachment and biofilm formation mediated by the Csu pili chaperone-usher assembly system (Jeon *et al.* , 2008 and Tomaras *et al.* 2008). Hence, reduced expression of RstA may explain the increased levels of iron-, attachment- and biofilm-related proteins observed in AB211.

The expression of spot 30 (Table 5.4) increased 2.2-fold in AB210 and was identified as PhoU, part of the *pst-* (phosphate transporter) operon involved in phosphate transport across the membrane. PhoU acts as a transcriptional regulator that negatively regulates the *pho* regulon. PhoU and the *pst* system have previously been implicated as virulence factors for *Proteus mirabilis* urinary tract infections (Jacobsen *et al.* 2008), due to the association of the *pst* system and the regulation of biofilm formation under phosphate limited conditions.

The expression of Spot 15 (Table 5.4) increased 3.1-fold in AB210 and identified as CsuD, part of the chaperon-usher pilus-assembly system (Tomaras *et al.* 2008) and is required for pilus-mediated motility (Siroy *et al.* 2006). The reason for its increased expression in AB210 when related proteins CsuA/B and PapC were identified as increased in AB211 is unknown. As little is known about the functions of the individual Csu subunits, CsuD may have other roles aside from pilus formation.

Overall, the number of differentially-expressed proteins clustered in this functional group, combined with their specific functions, suggests that AB211 may be more adept at cell attachment and/or biofilm formation than AB210.

Spot no.	Protein ID	GI number	Mol Wt.	No. unique peptides	Fold expression increase *vs.* AB211	*p* value ANOVA
12	LysM domain/BON superfamily protein	gi|169634111	17 kDa	6	5.4	**0.001**
13	Aspartate/tyrosine/aromatic aminotransferase	gi|184158727	60 kDa	11	4.6	**0.042**
14	MinD cell division inhibitor a membrane ATPase activates MinC	gi|169634045	31 kDa	2	4.4	**$3.2e^{-4}$**
15	CsuD	gi|126642259	90 kDa	4	3.1	**$6.5e^{-4}$**
16	Glutathione S transferase	gi|260549986	23 kDa	2	3.1	**0.003**
17	Alkyl hydroperoxide reductase C22 subunit	gi|126641253	18 kDa	7	3.1	**0.002**
18	Superoxide dismutase	gi|50084526	23 kDa	4	2.9	**0.015**
19	Nucleoside diphosphate kinase	gi|215484688	15 kDa	6	2.8	**0.001**
20	Imidazole glycerol phosphate synthase subunit HisF	gi|169794437	28 kDa	8	2.8	**$3.4e^{-4}$**
21	Methylisocitrate lyase	gi|213155491	32 kDa	7	2.7	**0.001**
22	Inorganic diphosphatase	gi|262373918	19 kDa	2	2.6	**0.004**
23	Peptide methionine sulfoxide reductase	gi|126640528	14 kDa	3	2.6	**0.005**
24	Putative dienelactone hydrolase	gi|126643437	19 kDa	5	2.5	**0.003**
25	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	gi|215482092	35 kDa	3	2.4	**0.002**
26	D amino acid dehydrogenase small subunit	gi|215485053	46 kDa	7	2.4	**0.046**
27	Succinyl CoA ligase ADP forming subunit alpha	gi|215482498	31 kDa	9	2.3	**0.004**
28	Malate dehydrogenase	gi|126643040	32 kDa	6	2.3	**0.02**
29	Succinyl CoA ligase ADP forming subunit alpha	gi|215482498	31 kDa	6	2.3	**0.003**
30	High affinity phosphate uptake transcriptional repressor PhoU	gi|126640349	24 kDa	8	2.2	**0.017**
31	Two-component regulatory system response regulator	gi|50083946	27 kDa	6	2.2	**$9.7e^{-5}$**
32	3-ketoacyl-CoA thiolase	gi|169634579	41 kDa	4	2.1	**0.002**
33	Electron transfer flavoprotein subunit alpha	gi|215482574	31 kDa	7	2.1	**0.011**
34	Electron transfer flavoprotein subunit beta	gi|215482573	26 kDa	5	2	**0.017**
35	3-phosphoglycerate kinase	gi|184157910	41 kDa	7	2	**0.019**

Table 5.4 Proteins that were highlighted by SameSpots software as displaying increased expression in AB210 *vs.* AB211.

5.4.5 Other proteins with expression increases in isolate AB211

Two proteins showed expression increases of 4.9-fold and 3.9-fold in AB211 (spots 1 and 2 respectively, Table 5.3); spot 1 was identified as a putative outer membrane protein by BLASTp. Whereas spot 2 returned as a possible capsule assembly protein and a signal peptide (both E values = 0 by BLASTp). Capsule assembly proteins transport capsular polysaccharides across the outer membrane, playing an important role in virulence in *A. baumannii* (Russo *et al.* 2010).

Expression of two isoforms of the ATP-dependant protease Hsp100 were increased 3-fold and 2-fold in AB211 (spots 5 and 11 respectively, Table 5.3), indicating an increased response to stress. By BLASTp analysis these proteins were identified as variants of ClpB, which is involved less in protein degradation but more in disaggregation and reactivation of misfolded protein aggregates (Zolkiewski, 2006) caused by stressful conditions *e.g.* extreme pH, osmolarity or temperature. Differential expression of ClpB in isolate AB211 could have been caused by the increased expression of proteins which localise to the outer membrane.

Other protein expression increases in AB211 include elongation factor G, which was increased 2-fold (spot 10; Table 5.3); this protein catalyses the translocation of the tRNA-mRNA complex across the ribosome, allowing polypeptide chain elongation to occur. Methionine synthase (cobalamin-binding subunit MetH) was increased 2.8-fold (spot 6; Table 5.3); it synthesises methionine from homocysteine via a vitamin B12-dependant pathway. The triggers for the increase in these particular enzymes and their biological significance are unclear.

5.4.6 Other proteins with expression increases in isolate AB210

The LysM domain/BON superfamily protein (spot 12; Table 5.4) showed a 5.4-fold expression increase in isolate AB210. The LysM or lysin domain contains a peptidoglycan-binding motif, present in many proteins capable of cell wall degradation. The BON superfamily consists of proteins containing the BON (Bacterial OsmY and nodulation) domain, as found in *e.g.* OsmY, an osmotic-shock-resistance protein. The BON domain is thought to interact with phospholipid membranes (Yeats & Bateman, 2003).

MinD (spot 14, Table 5.4) is involved in the inhibition of FtsZ cell division proteins (the Z ring); it activates the inhibitor, MinC and directs the site of septum formation for cell division, ensuring it initiates at mid-cell (Lutkenhaus, 2007). Expression of MinD was increased 4.4-fold in AB210, which indicates that AB211 may divide at a slower rate than AB210.

The higher levels of these metabolic proteins in AB210 could mean a higher level of energy generation, which could increase the production of free-radicals as respiration by-products relative to AB211. Consistent with this are the observed increases in expression of antioxidant proteins glutathione S transferase (spot 16, Table 5.4; 3.1-fold increase), alkyl hydroperoxide reductase subunit C (spot 17, Table 5.4; also a 3.1-fold increase) and superoxide dismutase (SOD; spot 18, Table 5.4; 2.9-fold increase) in AB210 as a possible countermeasure to greater levels of respiration. The remaining proteins (n = 16, Table 3) that were all down-regulated in AB211 all had roles in carbohydrate and amino acid metabolism. Further work is needed to determine the significance of these proteins.

5.5 DIGE comparison of the post-therapy isolate (AB211) *vs.* the laboratory mutant (AB210-6)

After initially comparing the pair of clinical isolates, the post-tigecycline therapy clinical isolate AB211 was compared with the laboratory mutant AB210-6, created by subculturing AB210 in increasing concentrations of tigecycline (Hornsey *et al.* 2010a). The two isolates used acquired their resistance in different manners (*in vivo* and *in vitro*) and were selected for comparison in order to demonstrate which proteins were required by or affected by tigecycline resistance. The aim was to identify a 'core' set of proteins common to the Ade efflux-mediated resistances, which may be essential for this particular mechanism. Also, as AB210-6 has a greater tigecycline MIC than AB211 (64 mg/L *vs.* 16 mg/L), there was a possibility that proteins expressed at a higher level in AB210-6 could be used as indicators of the level of resistance.

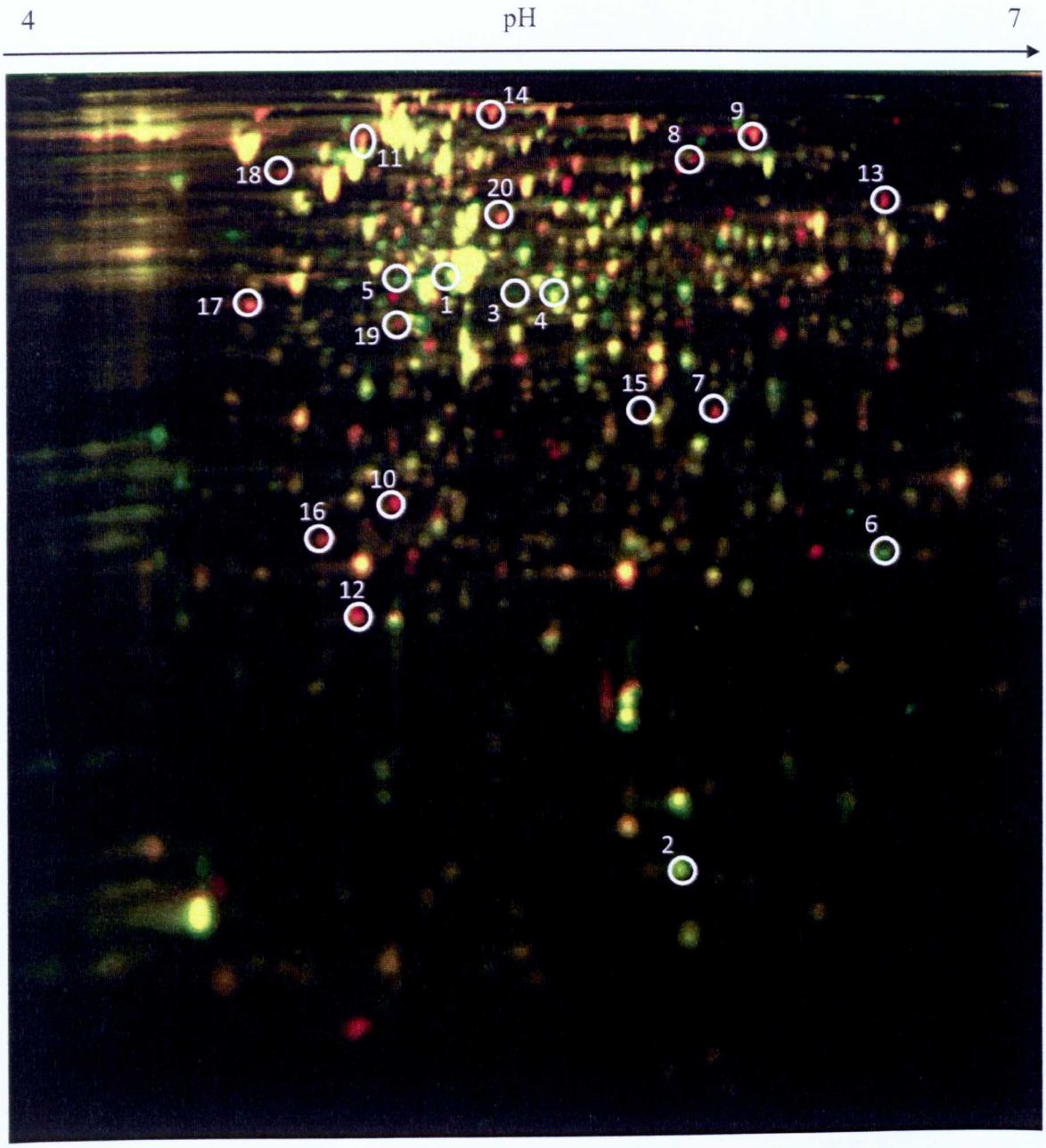

Figure 5.9. 2-D separation of DIGE-labelled proteins, using extracts from AB211 (green) and AB210-6 (red) separated over a pH gradient of 4-7 and through a 12% polyacrylamide gel. Numbers correspond to the identifications in table 5.5.

SameSpots analysis of the AB211 *vs.* AB210-6 DIGE gels identified 29 proteins as having differential expression between the two isolates (Fig. 5.9). Twenty of these proteins were successfully identified by LC-MS/MS, 14 of which showed increased expression in isolate AB210-6, while six showed increased expression in isolate AB211.

5.5.1 Proteins identified as increased in AB211 *vs.* AB210-6

The expression of polysaccharide biosynthesis protein (Spot 1; Table 5.5) increased 2.5-fold in AB211. A similar protein was highlighted previously in the comparison between AB210 and AB211 as present in AB210 but absent in AB211 (section 5.4.1 table 5.2). Its appearance suggests that expression of the polysaccharide synthesis protein in AB210 and not AB211 was not a unique event and highlights the difficulties of making inferences on the basis of spot presence/absence.

The acyl-CoA dehydrogenase protein (Spot 3; Table 5.5) also showed a 2.2-fold increase in expression in AB211. It was identified as an AcdB-like protein, which utilises long-chain fatty acids for energy metabolism. *Acinetobacter* is known to produce a waxy ester as an energy storage molecule, synthesised from an alcohol and an acyl-CoA. It is possible that an increase in this protein could mean that AB211 is more adapted to energy storage than AB210-6 (Tani *et al.* 2002)

The DegT/DnrJ/EryC1/StrS aminotransferase family protein (Spot 4; Table 5.5) displayed an expression increase of 2.1-fold, the products of this family are involved in the biosynthesis of sugar portions of cell-surface polysaccharides (Shoji *et al.* 2002). The increased expression of this protein plus the increased expression of the polysaccharide biosynthesis protein (Spot 1; Table 5.5) shows that AB211 may better equipped to form biofilms than AB210-6 as well as AB210.

DNA-directed RNA polymerase subunit alpha (Spot 5; Table 5.5) increased 2.1-fold in expression, this protein is a key element in gene transcription, mediating the interactions between RNA polymerase, transcription factors and DNA. Subunit alpha associates with such regulators as MarA and SoxS to activate a wide variety of genes (Dangi *et al.* 2004).

Spot no.	Identification	GI number	Mol. Wt.	No unique peptides	Fold difference in relative expression *vs.* AB210-6	P values (ANOVA)
1	Polysaccharide biosynthesis protein	gi|169632087	**39 kDa**	**8**	**2.5**	**0.013**
2	30S ribosomal protein S6	gi|50085515	**15 kDa**	**2**	**2.2**	**0.002**
3	Acyl-CoA dehydrogenase protein (AcdB-like)	gi|169633254	**41 kDa**	**3**	**2.2**	**0.02**
4	DegT/DnrJ/EryC1/StrS aminotransferase family protein	gi|332873567	**43 kDa**	**6**	**2.1**	**0.013**
5	DNA-directed RNA polymerase subunit alpha	gi|50086190	**37 kDa**	**12**	**2.1**	**0.009**
6	3-oxoacyl-[acyl-carrier-protein] reductase	gi|169634113	**26 kDa**	**4**	**2**	**$3e^{-4}$**
7	Enoyl-CoA hydratase/carnithine racemase	gi|184157662	**29 kDa**	**6**	**-3**	**0.013**
8	Acetyl/propionyl-CoA carboxylase subunit alpha	gi|184157591	**62 kDa**	**7**	**-2.7**	**0.004**
9	Multifunctional fatty acid oxidation complex subunit alpha	gi|169634578	**78 kDa**	**18**	**-2.6**	**0.004**
10	Putative antioxidant protein	gi|126642887	**19 kDa**	**4**	**-2.6**	**0.003**
11	Oligopeptidase A	gi|213158799	**77 kDa**	**19**	**-2.5**	**0.022**
12	LysM domain/BON superfamily protein	gi|169634111	**17 kDa**	**8**	**-2.5**	**0.006**
13	3-hydroxyacyl-CoA dehydrogenase	gi|184157663	**57 kDa**	**11**	**-2.2**	**0.045**
14	Aconitase A	gi|184156882	**100 kDa**	**17**	**-2.2**	**0.022**
15	Cell division inhibitor, a membrane ATPase, activates MinC	gi|169634045	**31 kDa**	**5**	**-2.1**	**0.026**
16	Glutathione S-transferase	gi|262279679	**23 kDa**	**6**	**-2.1**	**0.002**
17	Transaldolase B	gi|184158577	**37 kDa**	**17**	**-2**	**0.008**
18	Chaperonin GroEL	gi|126642698	**50 kDa**	**9**	**-2**	**0.018**
19	Outer membrane protein A	gi|126642864	**37 kDa**	**2**	**-2**	**0.027**
20	NAD-dependent aldehyde dehydrogenase	gi|184158978	**51 kDa**	**13**	**-2**	**0.004**

Table 5.5 Proteins that were highlighted by SameSpots software as displaying differential expression between AB210-6 and AB211.

The 3-oxoacyl-acyl-carrier-protein reductase (Spot 6; Table 5.5) showed a 2-fold increase in expression and is an essential protein involved in fatty acid biosynthesis. The protein is coded for by the fabG gene and as part of the fatty acid synthase multienzyme complex, it catalyses an essential step in fatty acid elongation. FabG has been speculated as a potential antimicrobial target due to the specificity of the reaction and the conserved sequence and ubiquity of the enzyme (Kristan *et al.* 2009).

5.5.2 Proteins identified as increased in AB210-6 *vs.* AB211

5.5.2.1 Proteins involved in lipid metabolism

The following proteins all displayed increased expression in AB210-6 and seemed to participate in lipid metabolism. For instance, the alpha subunit of the multifunctional fatty acid oxidation (MFAO) complex (Spot 9; Table 5.5) displayed a 2.6-fold increase in expression and returned as FadB gene product by BLASTp (E value = 0). Enoyl-CoA hydratase (Spot 7; Table 5.5) displayed an expression increase of 3-fold and returned as PaaB by BLASTp analysis (E value = 0). 3-hydroxylacyl-CoA dehydrogenase (Spot 13; Table 5.5) showed a 2.2-fold increase in expression, it also had high similarity to the paaC gene product by BLASTp analysis (E value = 0). These latter two proteins are known to be associated with the MFAO complex (Yangs & Elzinga, 1993).

Also included in this group are acetyl/propionyl-CoA carboxylase subunit alpha (Spot 8; Table 5.5) which showed an expression increase of 2.7-fold in AB210-6 (returned as biotin carboxylase *accA* gene by BLASTp; E value = 0). NAD-dependant aldehyde dehydrogenase (Spot 20; Table 5.5) showed an expression increase of 2-fold and showed high similarity to phenylacetaldehyde dehydrogenase (PAD) by BLASTp analysis (E value = 0). Some of the proteins in this group displayed high similarity to the *paa* (phenylacetic acid degradation) genes of *E. coli* which degrade aromatic compounds by converting them into phenylacetyl-CoA which can be catabolised into TCA intermediates. The Paa degradation pathway is also a common pathway for metabolism, implying that AB210-6 may be better at utilising phenylalanine for energy. It is also required for full pathogenicity for *Burkholderia cenocepacia* in a *Caenorhabditis elegans* infection model (Law *et al.* 2008), these same organisms showed a reduction in virulence when

these genes were knocked-out. However, the relevance of these changes to laboratory-acquired tigecycline resistance is unknown.

5.5.2.2 Increased expression of stress defence proteins in AB210-6

There were three proteins displaying increased expression which functioned as stress defence proteins. Expression of a putative antioxidant protein (Spot 10 ; Table 5.5) increased 2.6-fold in AB210-6 and returned as a oxidoreductase enzyme of the AhpC family by BLASTp analysis (E value = $4e^{-107}$).

Expression of glutathione S-transferase (Spot 16; Table 5.5) increased 2.1-fold in AB210-6. It catalyses addition of glutathione (GSH) group onto potentially harmful electrophilic compounds, 'quenching' their reactive groups and protecting cell from *e.g.* DNA damage. It has been highlighted as an important gene required for the intrinsic resistance of *A. baylyi* to multiple antibiotics, as inactivation of the glutathione gene *gshA* confers hypersusceptible phenotypes (Gomez & Neyfakh, 2006).

Expression of chaperonin GroEL (Spot 18; Table 5.5) increased 2-fold in AB210-6. This protein re-folds denatured or mis-folded proteins and has been shown to play a role in resistance to antibiotics and heat stress in *A. baumannii* (Cardoso *et al.* 2010).

5.5.2.3 Other proteins with increased expression in AB210-6

The LysM/BON superfamily protein (Spot 12; Table 5.5) showed a 2.5-fold expression increase in AB210-6. There have been reports that the LysM protein domain is required for binding the peptidoglycan layer to the membrane in Gram-positive (Frankel *et al.* 2012) and Gram-negative bacteria (Poggio, 2010). This has also been demonstrated to be true also for *Acinetobacter* sp. (Cabral *et al.* 2011). Proteins containing this domain were identified in all comparisons with AB211 where it is consistently reduced in expression.

Aconitase A or AcnA (Spot 14; Table 5.5) expression increased 2.2-fold in AB210-6, AcnA catalyses the interconversion of citrate and isocitrate in the TCA cycle, it may have

importance in iron regulation, growth, superoxide/radical sensitivity due to key function and its essential 4Fe-4S cluster (Varghese *et al.* 2003). AcnA is generally induced under stress conditions by SoxRS and regulated by Fur, it also has the ability to bind mRNA (Tang *et al.* 2005). AcnA is also reported to have post-transcriptional regulatory activity on flagellum synthesis in *Salmonella enterica* (Tang *et al.* 2004).

The cell division inhibitor (Spot 15; Table 5.5) expression increased by 2.1-fold in AB210-6, it is also known as septum site-determining protein MinD, part of the MinCDE operon which regulates cell division (Lutkenhaus, 2007). This protein has been highlighted before in the comparison of AB210/AB211.

Outer membrane protein A (Spot 19; Table 5.5) showed a 2-fold increase in expression. It is an important multifunctional protein and among its roles; OmpA is thought to anchor the outer membrane to the peptidoglycan layer (Park *et al.* 2012), mediate attachment to biotic surfaces/cells (Choi *et al.* 2008) and is essential for biofilm formation (Cabral *et al.* 2011).

5.6 DIGE comparison of laboratory mutant (AB210-6) with pre-therapy isolate (AB210)

The next comparison involved the pre-tigecycline therapy clinical isolate AB210 and its laboratory-generated mutant AB210-6. Because the two isolates are so similar, it was hoped that proteins required for tigecycline resistance could be highlighted and identified with less 'noise' *i.e.* fewer changes unrelated to the resistance mechanism. For instance, when comparing AB210 and AB211, there were many changes in protein expression which were not caused by the acquisition of resistance but by unrelated genetic mutations (Hornsey *et al.* 2011). In this comparison, it was thought that the only factor differing between the two isolates is the resistance mechanism, so any differentially-expressed proteins were more likely to be an effect of tigecycline resistance.

SameSpots highlighted 23 protein spots as displaying differential expression between AB210-6 and AB210 (Fig. 5.10). However, on this occasion, only 7 identifications were returned by LC-MS/MS; three proteins displayed increased expression in AB210 and four showed increased expression in AB210-6. It is unclear why such a small proportion of proteins returned identifications by LC-MS, although as the excised gel spots were stored at -80°C for extended

periods and subjected to some degree of freezing and thawing, it is possible that some protein degradation occurred.

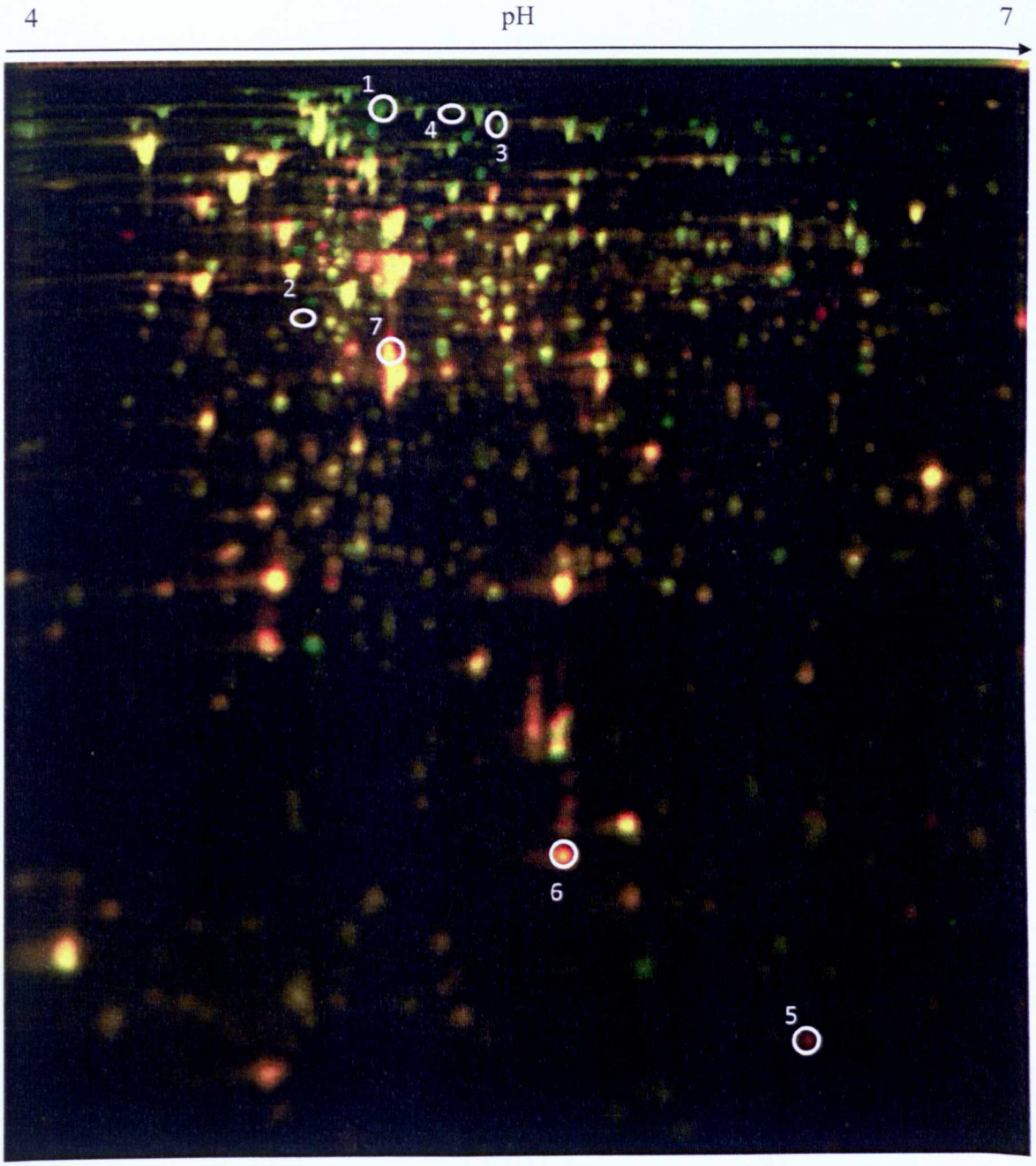

Figure 5.10 2-D separation of DIGE-labelled proteins using extracts from AB210-6 (green) and AB210 (red) separated over a pH gradient of 4-7 and through a 12% polyacrylamide gel. Numbers correspond to the proteins in table 5.6.

5.6.1 Proteins identified as increased in AB210-6

Most of the proteins highlighted in this comparison as differentially regulated have been seen in earlier comparisons of these isolates. While this suggests similar mechanisms are producing upregulated AdeAB both *in vitro* and *in vivo*, too few proteins were identified in this comparison to make reliable mechanistic inferences.

The ATP-dependent protease, Hsp 100 (spot 1; Table 5.6) increased in expression 2.8-fold in AB210-6 and was identified as a variant of ClpB by BLASTp analysis (E value = 0). ClpB is involved less in protein degradation, but more in disaggregation and reactivation of misfolded protein aggregates (Zolkiewski, 2006). This protein has been identified previously as increased in AB211 (see section 5.4.4), possibly due to the increased expression and trafficking of other proteins such as BfrD and YaeT to the outer membrane.

The Holliday junction DNA helicase RuvB (spot 2; Table 5.6) showed an expression increase of 2.5-fold in AB210-6. RuvB is part of the *ruv* operon, encoding homologous recombination proteins that make up the resolvase complex, which processes holliday junctions formed during genetic recombination (Zhang *et al.* 2010). These Ruv proteins also participate in mutation repair due to the similar enzyme activities required and may provide AB210-6 with greater protection against DNA damage than AB211.

The expression of malate synthase G (spot 3; Table 5.6) increased 2.2-fold in AB210-6 and catalyses the formation of malate and coenzyme A (CoA) from acetyl-CoA and glyoxylate. This allows the bypass of the TCA cycle by permitting growth on acetyl-CoA sources (*e.g.* lipids). It is thought that this glyoxylate bypass facilitated by malate synthase G is of high importance to pathogenesis in *e.g. Mycobacteruium tuberculosis* and *Pseudomonas aeruginosa* by allowing growth on host-derived lipids to increases chances of survival (Roucourt *et al.* 2009).

Spot no.	Identification	GI number	Molecular weight	No. unique peptides	Fold difference in relative expression *vs.* AB210	P values (ANOVA)
1	ATP-dependent protease, Hsp 100, part of multi-chaperone system with DnaK, DnaJ, and GrpE [*A. baumannii* SDF]	gi|169633163	95	29	+2.8	0.02
2	Holliday junction DNA helicase RuvB [*A. baumannii* SDF]	gi|169632719	37	8	+2.5	0.026
3	Malate synthase G [*A. baumannii* SDF]	gi|169633457	80	6	+2.2	0.012
4	Alanyl-tRNA synthetase [*A. baumannii* ATCC 17978]	gi|126641224	92	5	+2.1	0.01
5	Signal peptide [*A. baumannii* AYE]	gi|169795430	13	2	-2.3	0.014
6	Nucleoside diphosphate kinase (NDK) [*A. baumannii* SDF]	gi|169634408	16	6	-2.2	0.011
7	Elongation factor Ts [*A. baumannii* ATCC 17978]	gi|126642362	31	12	-2.1	0.047

Table 5.6 Identifications of proteins highlighted as displaying differential expression between isolates AB210 and AB210-6

Alanyl-tRNA synthetase (spot 4; Table 5.6) expression increased 2.1-fold in AB210-6 and catalyses the attachment of alanine to its corresponding tRNA for delivery to the ribosome. tRNA synthetases can have alternative functions such as modification of cell peptidoglycan (Villet *et al.* 2007), although the significance of alanyl-tRNA synthetase differential regulation here is unknown.

5.6.2 Proteins identified as increased in AB210

The proteins displaying increased expression in AB210 compared to AB210-6 include a signal peptide (spot 5; Table 5.6) which increased in expression 2.3-fold and was confirmed a signal peptide by Signalp 4.0 (http://www.cbs.dtu.dk/services/SignalP/). This protein also has an OB-fold (or Bacterial OB-fold/BOF), found via BLASTp, which is known to be common in nucleic-acid binding domains (Ginalski *et al.* 2004). Interestingly, in the comparison between AB210 and AB211, an OB-fold-containing protein was detected as unique to AB210. These independent comparisons show that the upregulation of the AdeAB efflux pump can result in lower levels of periplasmic BOF proteins, however the exact function of these proteins is unknown.

Nucleoside diphosphate kinase or NDK (spot 6; Table 5.6) showed a 2.2-fold increase in expression in AB210 compared with AB210-6. Strains of *E. coli* lacking this protein have shown elevated mismatch mutation rates and in NDK/MutS double mutants this rate is increased further, NDK deficiency is thought to stimulate replication errors by DNA polymerase (Miller *et al.* 2002). Hornsey *et al.* (Hornsey *et al.* 2011) showed via whole-genome sequencing that AB211 had a mutation in the *mutS* gene which could have led to the high number of mutations found in this strain. The results from the earlier AB210/AB211 comparison show that NDK is also higher in AB210 than AB211. It is unknown whether the differential regulation of NDK or the *mutS* mutation occurred first *i.e.* whether one change causes the other.

Elongation factor Ts or EF-Ts (spot 7; Table 5.6) expression increased in AB210 2.1-fold, EF-Ts is involved in polypeptide synthesis and functions by stimulating the binding of aminoacyl-tRNA to the ribosome. EF-Ts has a role as a stress-induced protein in *E. coli,* acting as a chaperone to enhance protein folding (Han *et al.* 2007).

5.7 Comparison of AB211 *vs.* AB211Δ*adeB*

The aim of this comparison was to try and determine which proteins/processes were affected by the regulation of AdeAB and which could be attributed to natural differences in the isolates (*e.g.* If a protein increased expression in AB211 *vs.* AB210 and then displayed reduced expression in AB211Δ*adeB*, its differential expression was likely an effect of AdeABC regulation). Multiple changes in protein expression were expected here due to the number of processes affected by the knockout of a key protein. But, if an expression pattern was found which is similar to any of the other comparisons *e.g.* protein expression increased in tigecycline-resistant isolates and reduced in -susceptible isolates, proteins/processes directly affected by pump upregulation may be elucidated.

There were originally 64 spots highlighted as differentially expressed between the two isolates, however, after digestion and LC-MS/MS analysis, only 39 were successfully identified (Fig. 5.11, Tables 5.7 and 5.8); 14 displayed increased expression in AB211 and 25 showed increased expression in AB211Δ*adeB*. As discussed earlier, there could be many reasons for this including; a degree of protein degradation due to storage of the excised gel spots, the stringency of the small *Acinetobacter* sp. database, or insufficient levels of peptide were eluted from the gel plug for LC-MS/MS identification.

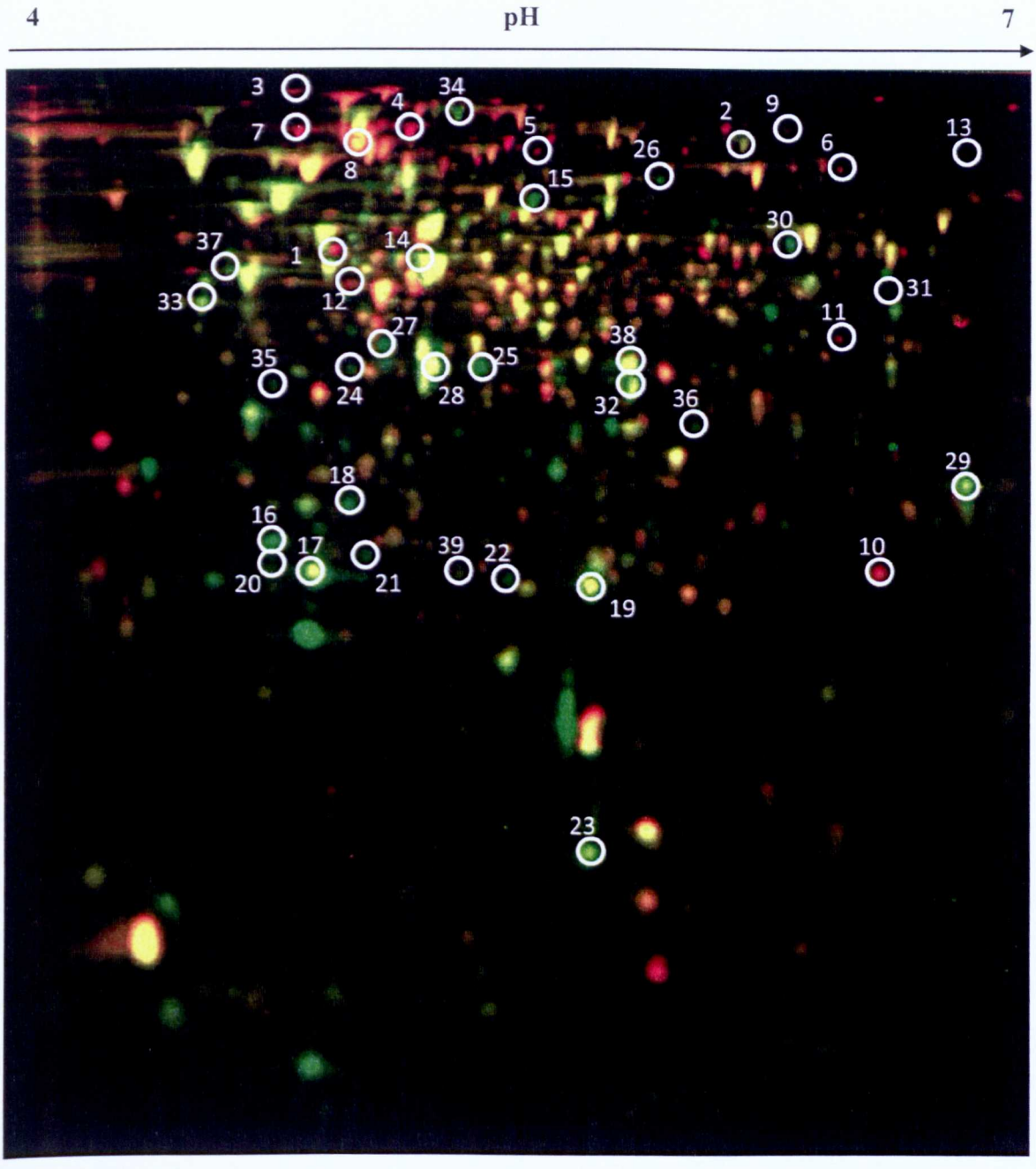

Figure 5.11. 2-D separation of DIGE-labelled proteins using extracts from AB211Δ*adeB* (green) and AB211 (red) separated over a pH gradient of 4-7 and through a 12% polyacrylamide gel. Numbers correspond to the proteins in tables 5.7 and 5.8.

5.7.1 Proteins increased in AB211

Multifunctional fatty acid complex subunit alpha (Spots 1 and 2; Table 5.7) expression was increased 4.4- and 3.6-fold respectively in AB211. Also known as FadB, this protein was discussed earlier in section 5.5.

B12 dependent methionine synthase or MetH (Spot 3; Table 5.7) expression increased 3-fold in AB211. This protein also increased expression in AB211 relative to AB210 (Table 5.3). Hondorp *et al.* showed that B12 *independent* methionine synthase is inactivated by oxidative stress (Hondorp & Matthews, 2004). As AB211 has lower levels of stress defence proteins compared to AB211Δ*adeB,* it may be possible that MetH expression increased to counteract the reduction in MetE to continue providing the cell with methionine. Also, RNAP alpha subunit is a known transcriptional activator of MetH and its increased expression may explain that of MetH (Fritsch *et al.* 2000).

ATP-dependent protease Hsp100 (Spot 4; Table 5.7) was previously identified as the chaperone ClpB and here expression was increased 3-fold. This protein has been previously identified as increased in AB211 and in AB210-6 (both tigecycline resistant isolates), suggesting its expression is increased in response to efflux upregulation.

Ferrichrome-iron receptor protein or FhuA (Spot 5; Table 5.7) expression increased 2.6-fold in AB211. FhuA expression also increased in AB211 compared to AB210 and its reduced expression in AB211Δ*adeB* implies that its increase is caused by AdeABC upregulation.

Succinate dehydrogenase flavoprotein subunit (Spot 6; Table 5.7) expression increased 2.4-fold in AB211. Succinate dehydrogenase expression is known to increase in biofilms (Gaupp *et al.* 2010) and Sdh is the main linker enzyme between the TCA and the electron transport (respiration) chain, it is possible that Sdh expression is increased as a consequence of the energy requirement of upregulated AdeABC.

Spot no.	Protein ID	GI number	Mol wt.	No. unique peptides	Expression difference *vs.* AB211Δ*adeB*	*p* value (ANOVA)
1	Multifunctional fatty acid oxidation complex subunit alpha [*A. baumannii* SDF]	gi|169634578	78 kDa	18	4.4	**0.026**
2	Multifunctional fatty acid oxidation complex subunit alpha [*A. baumannii* AYE]	gi|169797435	78 kDa	14	3.6	**0.024**
3	B12-dependent methionine synthase [*A. baumannii* ACICU]	gi|184157253	136 kDa	9	3	**0.008**
4	ATP-dependent protease Hsp 100 [*A. baumannii* ATCC 17978]	gi|126641234	93 kDa	17	3	**0.028**
5	Ferrichrome-iron receptor protein [*A. baumannii* ACICU]	gi|184158352	78 kDa	7	2.6	**0.018**
6	Succinate dehydrogenase flavoprotein subunit [*A. baumannii* SDF]	gi|169632622	67 kDa	13	2.4	**0.011**
7	30S ribosomal protein S1 [*A. baumannii* ATCC 17978]	gi|126641617	53 kDa	2	2.3	**0.014**
8	Elongation factor G [*A. baumannii* SDF]	gi|169634057	79 kDa	18	2.1	**0.003**
9	Elongation factor G [*A. baumannii* SDF]	gi|169634057	79 kDa	5	2.1	**0.004**
10	Electron transfer flavoprotein beta-subunit [*A. baumannii* ATCC 17978]	gi|126642663	23 kDa	8	2.1	**0.007**
11	UDP-N-acetylenolpyruvoylglucosamine reductase, FAD-binding [*A. baumannii* SDF]	gi|169633753	40 kDa	5	2.1	**0.023**
12	DNA-directed RNA polymerase subunit alpha [*Acinetobacter* sp. ADP1]	gi|50086190	37 kDa	6	2	**0.003**
13	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA [*A. junii* SH205]	gi|262372168	69 kDa	8	2	**0.015**
14	Elongation factor Tu [*A. baumannii* ATCC 17978]	gi|162286746	41 kDa	4	2	**0.001**

Table 5.7. Proteins increased in expression in AB211 *vs.* AB211Δ*adeB*

Two elongation factors were identified as displaying increased expression in AB211: elongation factor G (Spots 8 and 9; Table 5.7) expression increased 2.1-fold for both spots while elongation factor Tu (Spot 14; Table 5.7) expression increased 2-fold. These elongation factors have additional chaperone activities similar to EF-Ts (see section 5.6.1); EF-Tu in particular possesses a wide range of functions from DNA repair to RNA processing (Caldas *et al.* 1998). EF-G and EF-Tu may act as chaperones to replace oxidative stress defence proteins, many of which were reduced in AB211 compared with AB211Δ*adeB*.

UDP-N-acetylenolpyruvoylglucosamine reductase or MurB (Spot 11; Table 5.7) expression increased 2.1-fold in AB211. MurB is involved in peptidoglycan turnover in the synthesis of the bacterial cell wall. Mur proteins are highly conserved and essential, the *murB* gene was found to be upregulated in biofilm-growing cells of *Leptospirilla* spp. (Moreno-paz *et al.* 2010).

DNA-directed RNA polymerase subunit alpha (Spot 12; Table 5.7) expression increased 2-fold in AB211. Interestingly, in every comparison featuring AB211, the RNAP alpha subunit consistently displayed increased expression in AB211, suggesting that it is a trait of the isolates rather than a consequence of upregulated AdeABC (expression was greater in AB211 than in AB210-6, even though tigecycline MIC is higher in AB210-6).

tRNA uridine 5-carboxymethylaminomethyl modification enzyme or GidA (Spot 13; Table 5.7) expression increased 2-fold in AB211. GidA was identified by Shin *et al.* as upregulated in *A. baumannii* biofilm cells (Shin *et al.* 2009) and functions by modifying tRNAs to prevent errors in gene expression, but also has activity as a global regulator and could aid in biofilm persistence, although its exact role in biofilms is unknown.

5.7.2 Proteins increased in AB211Δ*adeB*

5.7.2.1 Stress defence proteins

There were many more proteins with increased expression in AB211Δ*adeB* and this selection includes a variety of proteins involved in oxidative/stress defence. These included alkyl hydroperoxide reductase C22 subunit or AphC (Spots 17 and 20; Table 5.8), expression of which

increased 4.1- and 3.4-fold respectively in AB211Δ*adeB* and superoxide dismutase (Spot 19; Table 5.8) expression of which increased 3.6-fold in AB211Δ*adeB*. Both of these proteins were previously reduced in AB211 in comparison with AB210 (Table 5.4). The putative antioxidant protein (Spot 18; Table 5.8) expression increased 3.6-fold in AB211Δ*adeB*, this protein returned as peroxiredoxin by BLASTp analysis (E value = $2e^{-115}$) which is part of the AhpC family (listed above), this exact same protein was increased in AB210-6 compared with AB211.

Flavohaemoprotein (Spot 35; Table 5.8) expression increased 2-fold in AB211Δ*adeB* Flavohaemoproteins are thought to play a role in oxidative stress defence as they have been reported to protect *Pseudomonas aeruginosa* against reactive oxygen species or ROS (Koskenkorva-frank & Kalmo, 2003).

5.7.2.2 Proteins involved in metabolism

There were some enzymes with expression increases from the TCA cycle; aconitase A (Spot 34; Table 5.8) expression increased 2.2-fold fold in AB211Δ*adeB*. Three spots were identified as malate dehydrogenase (Spots 24, 27 and 28; Table 5.8) which increased 2.9-, 2.5- and 2.4-fold respectively in AB211Δ*adeB*. This protein consistently displayed reduced expression in all comparisons of AB211, even its knock out derivative, suggesting that this expression change is isolate specific rather than a consequence of efflux upregulation. Three spots were identified as succinyl-CoA synthetase, two as the alpha subunit (Spots 32 and 38; Table 5.8) which increased expression 2.3- and 2-fold respectively and one as the beta chain (Spot 37; Table 5.8) which showed a 2-fold expression increase. Succinyl CoA synthetase was not identified in any of the other comparisons, which suggested that its increased expression here may be purely due to the absence of active AcrABC. Succinate metabolism appears to be a significant factor in AdeABC upregulation, as succinate dehydrogenase was increased in AB211 and succinate CoA synthase was increased in its knockout mutant.

Spot no.	Protein ID	GI number	Mol wt.	No. of unique peptides	Expression difference *vs.* AB211	*p* value (ANOVA)
15	Aspartate aminotransferase [*A. baumannii* ACICU]	gi|184158727	60 kDa	14	-5.2	**0.031**
16	Enoyl-CoA hydratase/carnithine racemase [*A. baumannii* ACICU]	gi|184157662	29 kDa	6	-4.5	**0.004**
17	Alkyl hydroperoxide reductase C22 subunit [*A. baumannii* ATCC 17978]	gi|126641253	18 kDa	4	-4.1	**0.002**
18	Putative antioxidant protein [*A. baumannii* ATCC 17978]	gi|126642887	19 kDa	4	-3.6	**0.006**
19	Superoxide dismutase [Fe] [*A. baumannii* SDF]	gi|169632935	23 kDa	10	-3.6	**0.013**
20	Alkyl hydroperoxide reductase C22 subunit [*A. baumannii* ATCC 17978]	gi|126641253	18 kDa	3	-3.4	**0.017**
21	Nitroreductase [*A. baumannii* ACICU]	gi|184159209	23 kDa	7	-3.3	**0.010**
22	Hypothetical protein ACICU_03125 [*A. baumannii* ACICU]	gi|184159445	21 kDa	9	-3.2	**0.020**
23	Nucleoside diphosphate kinase (NDK) [*A. baumannii* SDF]	gi|169634408	15 kDa	5	-2.9	**0.003**
24	Malate dehydrogenase [*A. baumannii* ATCC 17978]	gi|126643040	32 kDa	4	-2.9	**0.012**
25	Coproporphyrinogen III oxidase [*A. baumannii* ACICU]	gi|184159627	36 kDa	8	-2.7	**0.008**
26	Peptide chain release factor 3 [*A. baumannii* ACICU]	gi|184156878	60 kDa	7	-2.7	**0.017**
27	Malate dehydrogenase [*A. baumannii* AYE]	gi|169794650	35 kDa	2	-2.5	**0.014**
28	Malate dehydrogenase [*A. baumannii* AYE]	gi|169794650	35 kDa	2	-2.4	**0.003**
29	Electron transfer flavoprotein beta-subunit [*A. baumannii* SDF]	gi|169632681	26 kDa	4	-2.4	**0.006**
30	Phosphoglucosamine mutase [*A. baumannii* SDF]	gi|169634755	48 kDa	11	-2.4	**0.013**
31	Methylcitrate synthase [*A. baumannii* AYE]	gi|169797717	44 kDa	11	-2.4	**0.020**
32	Succinyl-CoA synthetase subunit alpha [*A. baumannii* SDF]	gi|169632628	31 kDa	8	-2.3	**0.008**
33	Transaldolase B [*A. baumannii* ACICU]	gi|184158577	36 kDa	2	-2.3	**0.010**
34	Aconitase A [*A. baumannii* ACICU]	gi|184156882	100 kDa	15	-2.2	**$5e^{-4}$**
35	Flavohaemoprotein (haemoglobin-like protein) [*A. baumannii* AYE]	gi|169794589	29 kDa	5	-2	**$7e^{-4}$**
36	Enoyl-CoA hydratase/carnithine racemase [*A. baumannii* ACICU]	gi|184157662	29 kDa	6	-2	**0.005**
37	Succinyl-CoA synthetase beta chain [*A. baumannii* ATCC 17978]	gi|126642749	37 kDa	6	-2	**0.006**
38	Succinyl-CoA synthetase subunit alpha [*A. baumannii* SDF]	gi|169632628	31 kDa	4	-2	**0.013**
39	O-methyl transferase [*A. baumannii* SDF]	gi|169633180	22 kDa	5	-2	**0.027**

Table 5.8. Proteins displaying increased expression in AB211Δ*adeB* compared with AB211

Transaldolase B or TalB (Spot 33; Table 5.8) expression increased 2.3-fold in AB211Δ*adeB*, while the other proteins in this group belong to the TCA cycle, TalB is part of the pentose phosphate pathway (PPP). Due to the reactions it catalyses, TalB is an important link between the PPP and glycolysis. Expression of both aconitase and TalB proteins also increased in AB210-6 in comparison with AB211, suggesting that expression of TCA cycle enzymes in AB211 is reduced.

5.7.2.3 Other proteins displaying expression increases

Enoyl-CoA hydratase/carnithine racemase (Spots 16 and 36; Table 5.8) expression increased 4.5- and 2-fold respectively in AB211Δ*adeB*, this protein is also known as PaaB, a phenylacetic acid degradation protein, which also showed increased expression in AB210-6 *vs.* AB211.

Hypothetical protein ACICU_03125 (Spot 22; Table 5.8) was returned as ATP:cob(I)alamin adenosyltransferase by BLASTp analysis (E value = $2e^{-137}$) and its expression increased 3.2-fold in AB211Δ*adeB*. This protein synthesises coenzyme B12 (adenosylcobalamin) from regular vitamin B12 (cobalamin) (Mera & Escalante-Semerena, 2011). It is interesting that AB211 displayed increased expression of MetH, yet here upon removal of the AdeABC efflux pump AB211Δ*adeB* appears to be utilising cobalamin. There could be a potential role of cobalamin in the upregulation of AdeABC efflux pump.

Nucleoside diphosphate kinase (Spot 23, Table 5.8) displayed a 2.9-fold expression increase in AB211Δ*adeB* and has been highlighted previously in section 5.6.2 as increased in AB210 compared with AB210-6. It also increased expression in AB210 compared with AB211 (Table 5.4), showing that it is lower in the resistant isolates compared with susceptible isolates.

5.8 Chapter Summary

In this study the expression patterns of four isolates of *A. baumannii* were compared using DIGE in order to detect changes related to resistance/susceptibility to tigecycline. When comparing AB210 and AB211, eight proteins were detected only in AB210 and five only in AB211. However, due to

the limited dynamic range of 2DGE it is not possible to provide a proof of absence for a particular protein and confirmation is required by other means *e.g.* genomics. The observed differences could be explained by large variations in abundance or posttranslational modifications. The genome sequences of AB210 and AB211 are available (Hornsey *et al.* 2011*)* which allowed corroboration of the proteomics findings with the genomics data. Two proteins confirmed as unique to AB210 were identified as AAC(6')-Ib, and MdaB and were absent from the genome of AB211. The AAC protein is an aminoglycoside resistance enzyme which acetylates the antibiotic and renders it ineffective and its absence in AB211 is consistent with the reduction in aminoglycoside MICs.

There were multiple proteins identified that could potentially confer an increased ability to sequester iron from the environment in AB211. These included the identification of AroD unique to AB211, whose catechol products can be used in iron acquisition. AB211 also displayed increases in BfrD (a catechol receptor) and a ferrichrome iron receptor protein, these three proteins taken together strongly suggest that AB211 would be better at scavenging iron than AB210 and may have a competition advantage *in vivo*. This potential *in vivo* advantage of AB211 is given further weight by the increased expression of proteins involved in pilus- and biofilm-formation and also capsule assembly. Ferrichrome iron receptor was also identified as displaying reduced expression in AB211Δ*acrB* compared with AB211, suggesting that AdeABC efflux pump regulation has a direct effect on the expression of ferrichrome iron receptor.

Overall, the majority of proteins with increased expression in AB211 were outer membrane proteins, while many of the proteins increased in AB210 were cytosolic and seemed to function in metabolism and oxidative stress defence (three antioxidant proteins all increased in AB210). These may be required due to lower levels of efflux than AB211, or because of an apparent increase in metabolic enzyme expression. From the protein profiles generated by this comparison, AB211 appeared potentially more virulent and may have a competitive advantage over AB210 under certain conditions *e.g.* low iron concentrations.

The additional comparisons of the clinical pair with the mutants provided extra insight into how differential regulation of the AdeABC pump affects the *A. baumannii* proteome. By comparing AB211 with a lab-mutant and an *adeB* knockout, expression of biofilm-forming proteins was

increased again in AB211. The polysaccharide biosynthesis protein combined with the presence of sugar-modifying enzyme for biofilm carbohydrate moieties provides more evidence that upregulated efflux causes expression increases in proteins that facilitate biofilm formation *e.g.* the DegT/DnrJ/EryC1/StrS aminotransferase family protein is involved in the biosynthesis of sugar portions of cell-surface polysaccharides (Shoji *et al.* 2002). There are other proteins increased in AB211 which support this same conclusion: that the tigecycline-resistant isolates with upregulated efflux are more adept at forming biofilms.

Although AB210 and AB210-6 were compared, not enough protein identifications were returned to make reliable inferences about the significance of proteins displaying differential expression. However, there were some proteins identified that have been seen in other comparisons: such as ClpB, NDK and malate synthase G.

The comparison of AB211 and AB210-6 revealed, amongst other changes, differences in the isolates' metabolism of lipids. AB211 displayed increased expression of proteins which together suggest an increase in the biosynthesis of fatty acids, possibly for energy storage. Whereas the proteins in AB210-6 suggest this isolate is more likely to utilise lipids for energy generation. This lipid catabolism could possibly be due to increased demand from TCA cycle for acetyl CoA (lipid metabolism would supply Acyl-CoA) which was suggested by Fernandez-Reyes *et al.* (Fernandez-Reyes *et al.* 2009). Alternatively, avoiding lipid catabolism could help control the cellular pH (generation of fatty acids would lower the pH). Malate synthase G, which increased in AB210-6 compared to AB210, provides further evidence for AB210-6 utilising lipids for energy. The tigecycline-susceptible isolates display more metabolic proteins with increased expression, AB211 particularly has consistently reduced expression of TCA cycle enzymes, suggesting it relies on alternative energy sources. This may explain the reduced expression of lipid-metabolising proteins which were found to be increased in AB210-6, to generate acetyl-CoA to feed the TCA cycle.

As fatty acids and other cellular metabolites are substrates of the AdeABC efflux pump, the expression of these proteins may increase to make up for the fatty acids being lost to increased efflux activity. Alternatively, in *E. coli* FadB is known to be consistently increased when grown in biofilms (Beloin *et al.* 2004) and as efflux pumps are known to significantly contribute to biofilm

formation (Matsumura *et al.* 2011) this increase in expression of this protein may be caused directly by upregulated AdeABC.

This needs further work utilising more clinical pairs of isolates to rigorously test these inferences and pin down whether any are in fact specific to the resistance mechanism or just isolate-specific changes unrelated to resistance. It is important to map these proteins onto their biological pathways to try and elucidate the subtle effects that differential efflux regulation has on a bacterial cell.

It was also observed that AB210-6 displayed higher expression of stress-defence proteins than AB211, which may contribute to AB210-6 being able to withstand higher tigecycline concentrations (64 mg/L *vs.* 16 mg/L in AB211). When comparing AB211 and its derivative knockout, the latter had more stress defence proteins with increased expression than AB211, emphasising how upregulated efflux protects the cell from stresses. ClpB was repeatedly identified in these DIGE comparisons; for instance it increased in AB211 in every comparison, as ClpB functions to re-fold misfolded proteins the upregulation of AdeABC may have detrimental effects on protein folding in AB211.

As mentioned earlier, NDK deficiency can stimulate replication errors in *E. coli.* A reduction in NDK expression may be advantageous to the resistant isolates by causing elevated rates of mutation under selection pressure. Strains of *E. coli* lacking this protein have shown elevated mismatch mutation rates and in NDK/MutS double mutants this rate is increased further, NDK deficiency is thought to stimulate replication errors by DNA polymerase (Miller *et al.* 2002). Hornsey *et al.* (Hornsey *et al.* 2011) showed via whole-genome sequencing that AB211 had a mutation in the *mutS* gene which could have led to the high number of mutations found in this strain. The results from the earlier AB210/AB211 comparison show that NDK was also higher in AB210 than AB211. It is unknown whether the differential regulation of NDK or the *mutS* mutation occurred first *i.e.* whether one change causes the other.

Due to the reduced production of metabolic proteins, proteins for lipid storage rather than utilisation, reduced stress defence proteins and increases in biofilm forming- and iron scavenging-related proteins, it appears that overall, AB211 seems set up for persistence. Many of the proteins

with expression increases in AB211 could confer a survival advantage under antibiotic therapy and potentially allow AB211 to outcompete its tigecycline-susceptible counterpart, AB210.

This work has contributed to the proteomic characterisation of *A. baumannii* by elucidating some of the effects of AdeABC differential regulation on the *A. baumannii* proteome *e.g.* NDK was consistently reduced in tigecycline-resistant isolates and increased in -susceptible isolates, ClpB was always increased in AB211 as was RNA polymerase subunit alpha, while Min cell division proteins, metabolic proteins and stress defence proteins were increased in tigecycline-susceptible isolates. While the original aim was to use proteomics to investigate the mechanism of resistance acquisition, the results were not able to answer this question. Instead this work shed light on the diverse changes in organism physiology and metabolism caused by the differential regulation of the AdeABC pump, including changes which may affect the virulence, persistence and recalcitrance of these isolates. By comparing the pre- and post-therapy *A. baumannii* clinical pair alongside derivative isolates, we can see patterns of protein expression begin to emerge. However, while DIGE has provided vast amounts of information about the test organisms, the techniques are not set up for high-throughput workflows as they are time consuming and require elaborate data analysis. A greater number of isolates need to be tested to confirm the reproducibility of the results, as confirmation of these observed differences in other *A. baumannii* pairs would allow us to draw more solid conclusions about the proteins involved in resistance. Nonetheless the DIGE technique is suitable for smaller scale analysis, revealing many changes in the tested clinical pair and its derivatives which would otherwise remain unobserved. This fact alone should warrant the use of proteomics in the analysis of unusual/complex resistance mechanisms/pathogens.

6. Results

Tigecycline resistance in *Enterobacter cloacae*

6.1 Background of isolates

To date, much work investigating *E. cloacae* and its antimicrobial resistances has been undertaken (Sanders & Sanders, 1997; Perez *et al.* 2007; Hornsey *et al.* 2010b). However, while it is understood that upregulation of the AcrABC efflux pump confers resistance to tigecycline in *E. cloacae* (Keeney *et al.* 2007 and Hornsey *et al.* 2010b); the consequences of this upregulation on the bacterial cell or whether there are any regulators/cofactors of the pump, are unknown. Here, the DIGE experimental approach (see methods section 2.10) was applied to investigate tigecycline resistance in *E. cloacae.* The lack of any previously published comparative proteomics studies on this clinically-relevant species makes this work all the more pertinent.

The clinical pair of isolates TGC-S and TGC-R were obtained from a patient before (TGC-S) and after (TGC-R) ciprofloxacin therapy. These isolates were selected to investigate the potential effects of differential levels of expression of the AcrABC efflux pump on the rest of the *E. cloacae* proteome. The antibiotic susceptibilities of the isolates were evaluated in AMRHAI at the HPA, where TGC-R was found to be resistant to both ciprofloxacin and tigecycline (both with an MIC of 4 mg/L) while TGC-S was susceptible to both compounds (with MICs of 0.5 mg/L each). TGC-R had been used to create an *acrB* gene knockout mutant, TGC-RΔ*acrB,* in which the gene was inactivated by the insertion of a gentamicin resistance cassette (Hornsey *et al.* 2010b), leaving TGC-RΔ*acrB* susceptible to tigecycline (MIC of 0.125 mg/L). As the level of efflux is increased in TGC-R compared to TGC-S and TGC-RΔ*acrB*, (Hornsey *et al.* 2010b) we hoped to identify proteins whose expression was affected by both the increase and decrease in efflux activity.

The aim was to identify proteins that may be involved in the efflux-mediated tigecycline resistance mechanism, with the additional objective of characterising the *E. cloacae* proteome. It is hoped that increased knowledge of the efflux resistance mechanism, its regulation and the effects of its differential regulation on *E. cloacae* cell physiology could contribute to the development of novel inhibitors/antagonists potentially capable of disabling efflux activity thus overcoming a broad and troublesome resistance mechanism.

6.1.1 2-Dimensional gel electrophoresis of *Enterobacter* extracts

All *E. cloacae* isolates were grown to late log phase in LB broth, cells were collected via centrifugation and lysed (as described in Methods section 2.5.2). The extracted proteins were quantified and separated by 2DGE using Immobilised pH gradients of 4-7 (see methods section 2.12.1). Each biological replicate (four in total) for each extract was optimised using 2DGE before any labelling with CyDyes due to availability of the dyes. These initial 2DGE separations were to demonstrate that; (i) the extracts were free from any charged or insoluble contaminants that could cause streaking and (ii) the proteins would separate with good resolution using the specified pH range. (see Fig. 6.1 Fig. 6.2 and Fig. 6.3). The gels displayed here yielded the highest number of resolved protein spots and hence, were used as 'picking gels' to supply the material needed for protein identification. The ProPic II scanner/picking robot used for this task could not visualise CyDye-labelled proteins, therefore SYPRO-stained gels were used for picking instead of the labelled originals. Although some gels displayed 'warping' caused by uneven acrylamide polymerisation, there was sufficient protein separation to use for spot-picking.

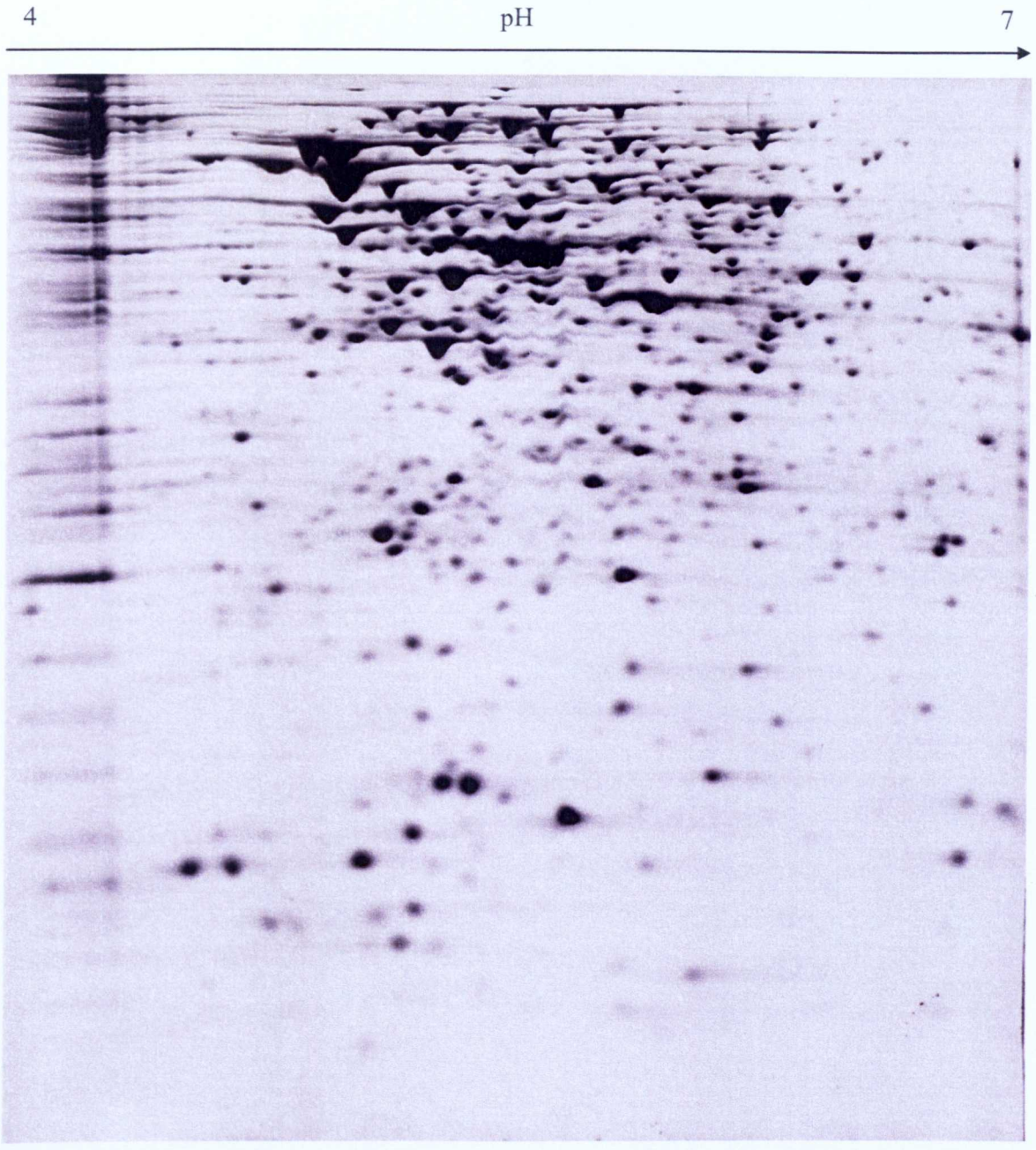

Figure 6.1 2DGE profile of TGC-S isolate. Total cell extract was separated using pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

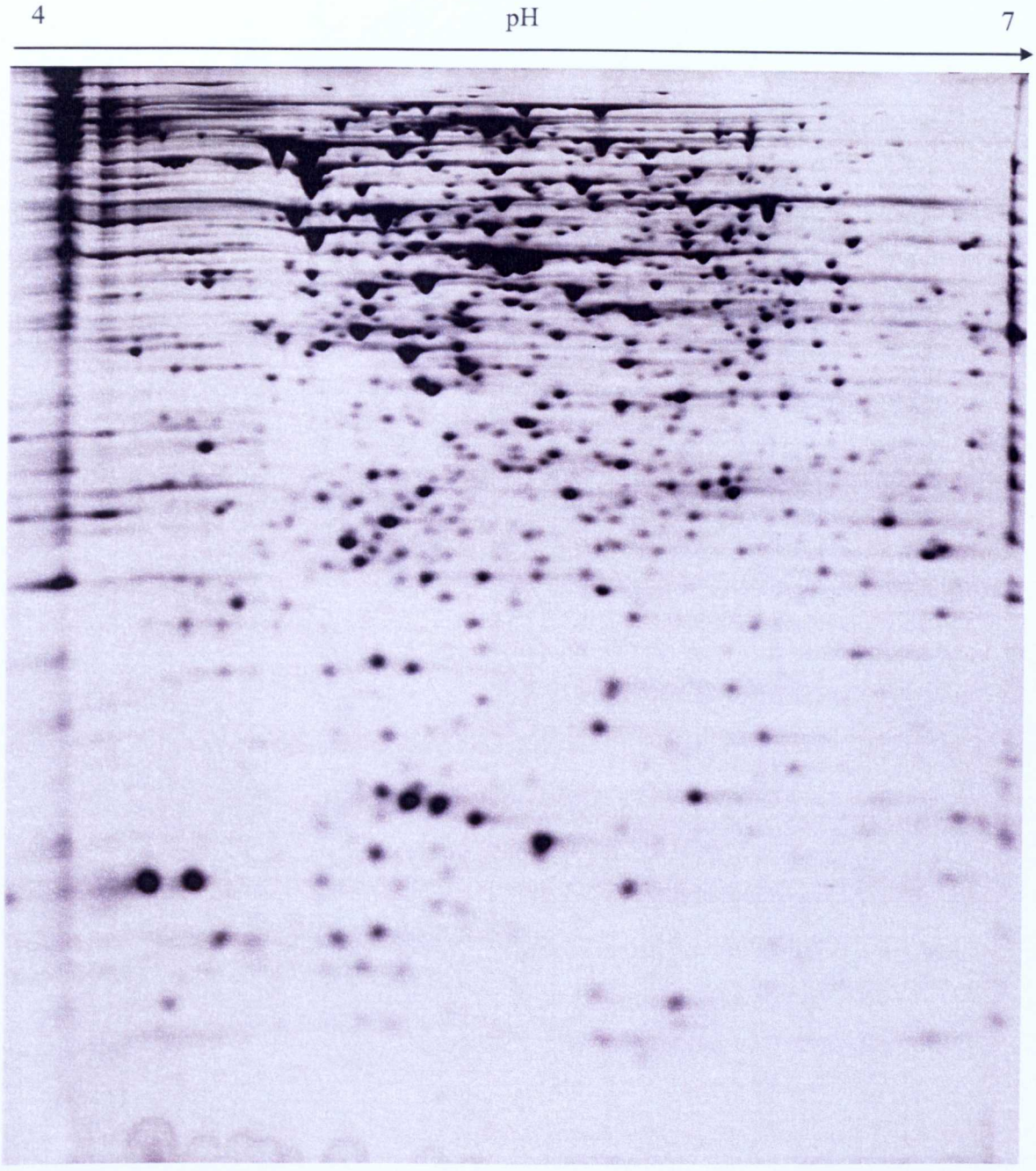

Figure 6.2 2DGE profile of TGC-R isolate. Total cell extract was separated using pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

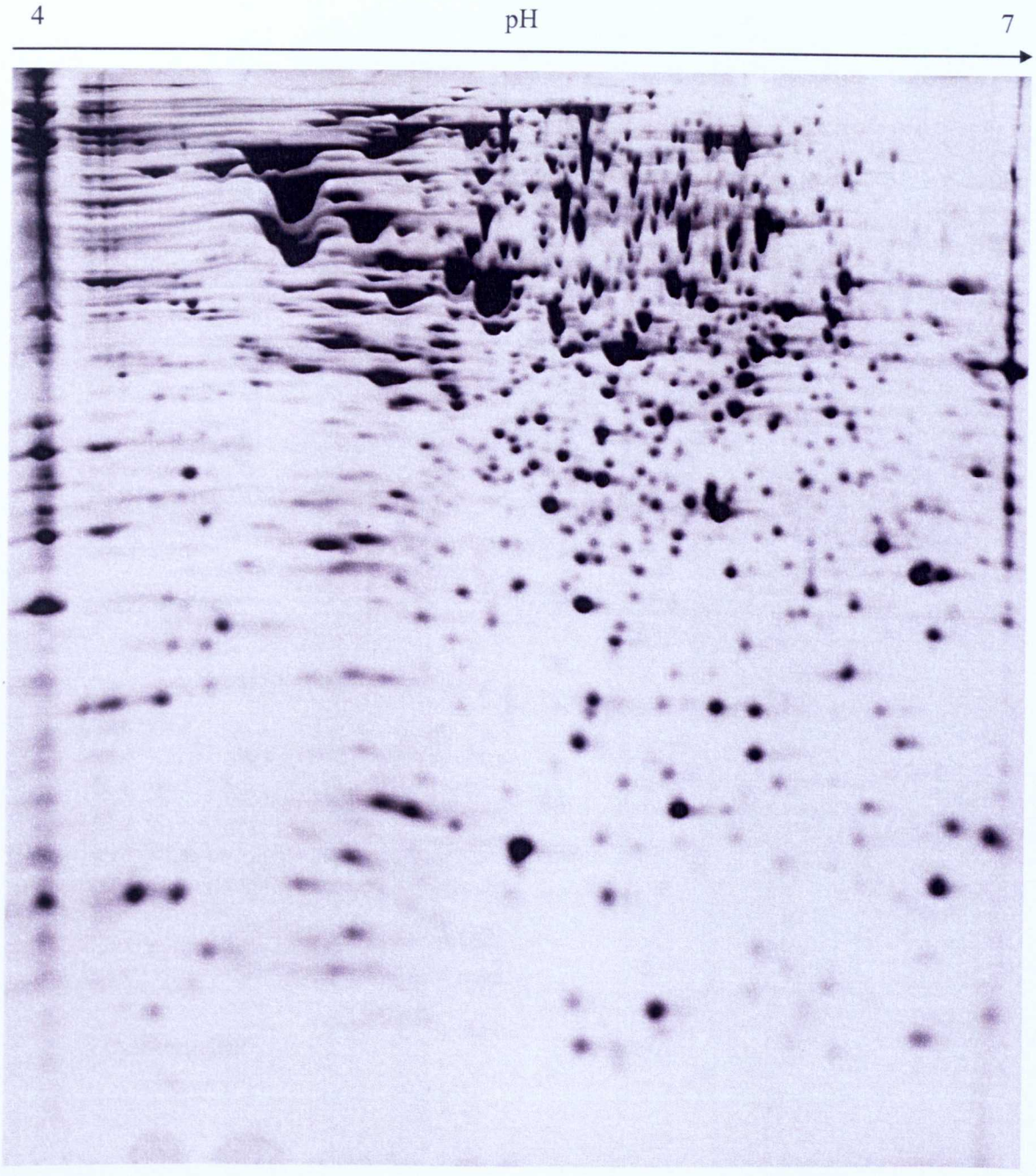

Figure 6.3 2DGE profile of TGC-RΔ*acrB* isolate. Total cell extract was separated using pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby and visualised using an Ettan Dalt imager (GE Healthcare).

2D DIGE gels were setup as described in Table 6.1 and gel images were analysed using the SameSpots software package (see methods section 2.14) from which c. 550 individual protein spots over the range of pH 4-7 were detected. Comparison of isolates TGC-S and TGC-R revealed 24 spots that were differentially expressed (Fig. 6.4). Of these, 21 were identified using LC-MS/MS (3 proteins did not return an identification), yielding 17 different proteins. Relative expression was greater for 6 spots and lower for 15 spots in tigecycline-resistant isolate TGC-R (Table 6.2). When isolate TGC-R was compared with mutant, TGC-RΔ*acrB* (Fig. 6.5) 26 differentially-expressed spots were highlighted: 23 of these spots returned identifications to give 21 different proteins, four spots showed greater relative expression in TGC-R and 17 had reduced expression in this isolate (Table 6.3). Most of the differentially-expressed proteins could be placed into the following two groups; (i) proteins which correlated directly with efflux expression *i.e.* increased with efflux pump up-regulation and decreased with efflux knockout and (ii) changes in protein expression with the potential to alter virulence. Further work needs to be done to characterise the remaining identified proteins.

Gel no.	Labelled with Cy3	Labelled with Cy5
1	TGC-S (4)	TGC-R (2)
2	TGC-RΔ*acrB* (3)	TGC-S (2)
3	TGC-R (1)	TGC-RΔ*acrB* (2)
4	TGC-S (3)	TGC-RΔ*acrB* (1)
5	TGC-RΔ*acrB* (4)	TGC-R (3)
6	TGC-R (4)	TGC-S (1)

Table 6.1 DIGE experimental setup for *E. cloacae* protein extracts with the biological replicate number in brackets

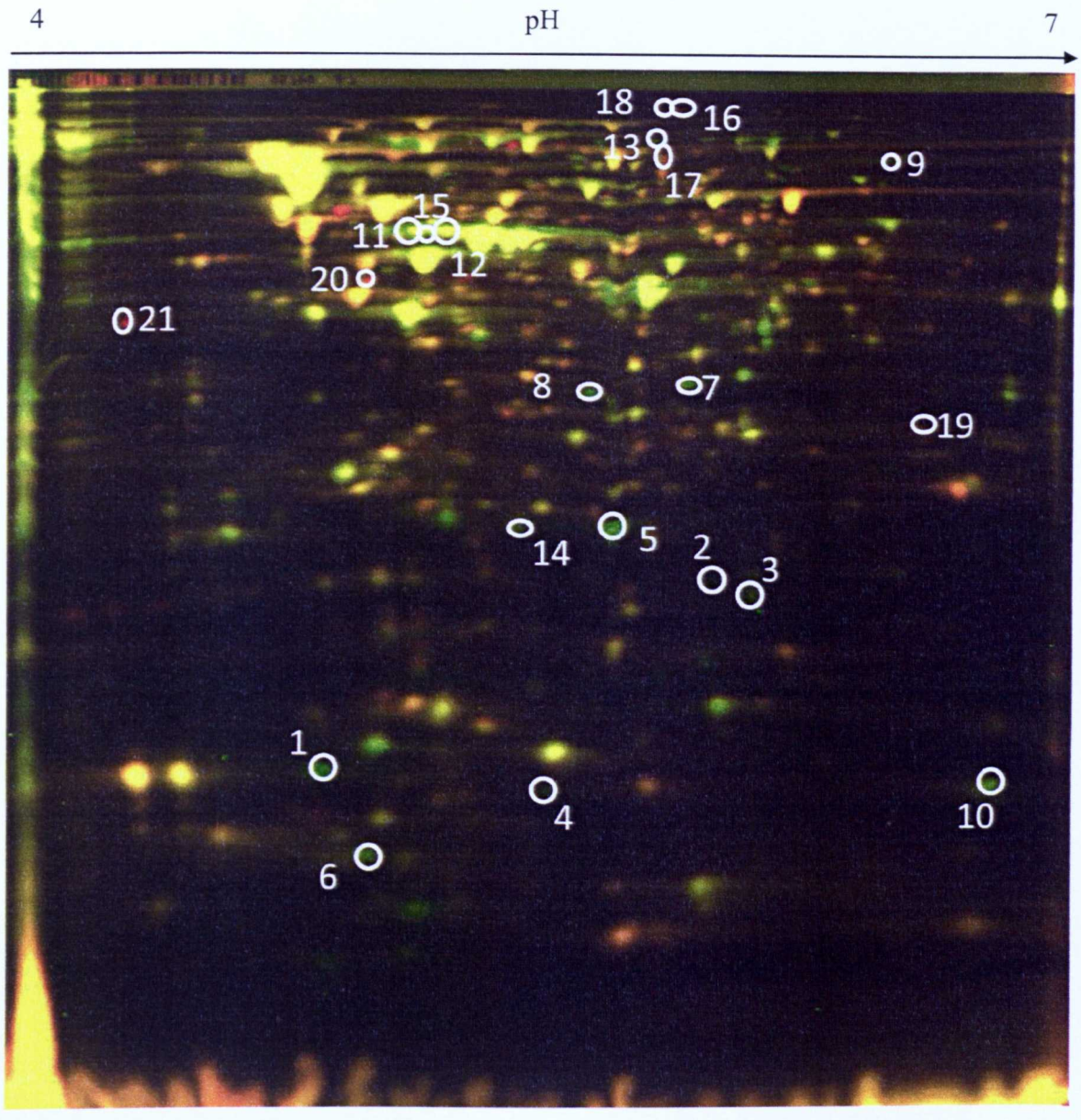

Figure 6.4 2-D DIGE image of TGC-R (Cy5 - red) *vs.* TGC-S (Cy3 - green). Green spots correspond to proteins from TGC-S, red spots correspond to proteins from TGC-R and yellow spots indicate that the protein is present in both isolates. Numbered, circled spots correspond to the identified proteins of interest in Table 6.2.

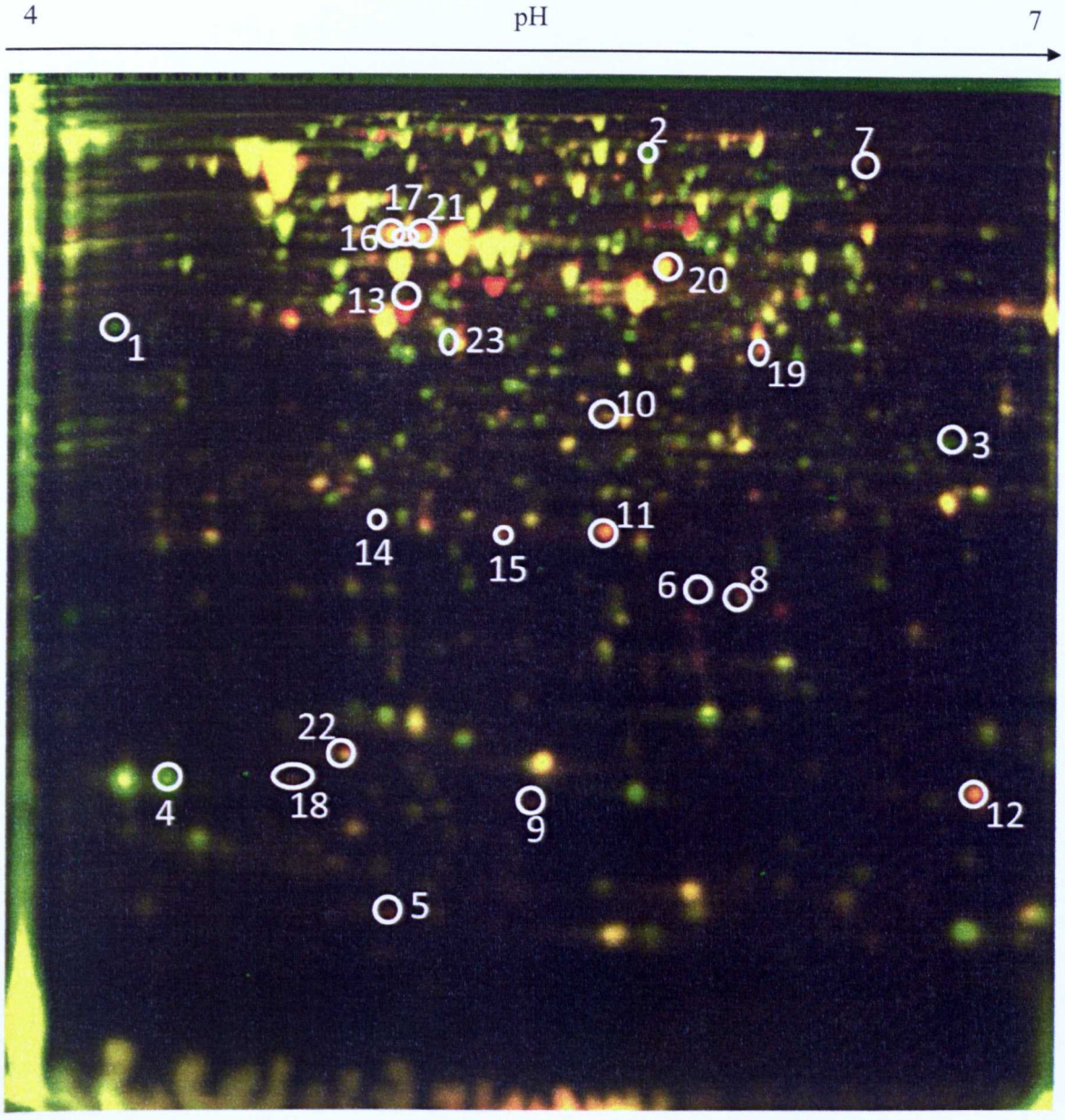

Figure 6.5 2-D DIGE image of TGC-R (Cy5 - red) *vs.* TGC-RΔ*acrB* (Cy3 - green). Green spots correspond to proteins from TGC-RΔ*acrB*, red spots correspond to proteins from TGC-R and yellow spots indicate that the protein is present in both isolates. Numbered, circled spots correspond to the identified proteins of interest in Table 6.3.

Spot no.	Protein ID	Mol. Wt.	GI number	No. unique peptides matched	Fold difference in expression *vs.* TGC-R	*p* VALUES (ANOVA)
1	Acid-induced glycyl radical enzyme [*Enterobacter cloacae* NCTC 9394]	14 kDa	gi|295097890	8	-4.6	$3.9e^{-4}$
2	DNA-binding ferritin-like protein (oxidative damage protectant) [*Enterobacter cloacae* NCTC 9394]	19 kDa	gi|295096504	6	-4.4	0.002
3	DNA-binding ferritin-like protein (oxidative damage protectant) [*Enterobacter cloacae* NCTC 9394]	19 kDa	gi|295096504	9	-2.9	$4.9e^{-4}$
4	Acid-induced glycyl radical enzyme [*Enterobacter cloacae* NCTC 9394]	14 kDa	gi|295097890	3	-2.9	0.001
5	Superoxide dismutase [*Enterobacter cancerogenus* ATCC 35316]	21 kDa	gi|261339584	3	-2.8	$2.5e^{-4}$
6	Acid-induced glycyl radical enzyme [*Enterobacter cloacae* NCTC 9394]	14 kDa	gi|295097890	7	-2.5	$2.5e^{-4}$
7	Septum site-determining protein MinD [*Enterobacter cloacae* NCTC 9394]	30 kDa	gi|295095589	6	-2.4	$1.7e^{-4}$
8	Aldo/keto reductases, related to diketogulonate reductase [*Enterobacter cloacae* NCTC 9394]	31 kDa	gi|295097529	10	-2.4	0.001
9	Pyruvate dehydrogenase [*Enterobacter cloacae* subsp. *cloacae* ATCC 13047]	62 kDa	gi|295057584	9	-2.3	0.001
10	Ribosomal subunit interface protein [*Enterobacter cancerogenus* ATCC 35316]	13 kDa	gi|288315682	3	-2.3	0.002
11	Isocitrate dehydrogenase [*Enterobacter cloacae* subsp. *cloacae* ATCC 13047]	46 kDa	gi|295057303	14	-2.2	$2.1e^{-4}$
12	Enolase [*Enterobacter cloacae* subsp. *cloacae* NCTC 9394]	46 kDa	gi|295097345	11	-2.1	0.011
13	Hypothetical protein ENTCAN_05473 [*Enterobacter cancerogenus* ATCC 35316]	85 kDa	gi|261339238	15	-2.1	0.005
14	Predicted periplasmic or secreted lipoprotein [*Enterobacter cloacae* NCTC 9394]	21 kDa	gi|295098511	5	-2.1	0.001
15	Enolase [*Enterobacter cloacae* subsp. *cloacae* NCTC 9394]	46 kDa	gi|295097345	10	-2	0.012
16	Pyruvate ferredoxin/flavodoxin oxidoreductase [*Enterobacter cloacae* SCF1]	62 kDa	gi|308748935	8	+2.4	0.003
17	Succinate dehydrogenase, flavoprotein subunit, *E. coli*/mitochondrial subgroup [*Enterobacter cloacae* NCTC 9394]	64 kDa	gi|295096570	9	+2.3	0.005
18	L-proline dehydrogenase /delta-1-pyrroline-5-carboxylate dehydrogenase [*Enterobacter cloacae* NCTC 9394]	144 kDa	gi|295096267	6	+2.2	0.005
19	Unnamed protein product [*Enterobacter cloacae*]	27 kDa	gi|219759156	8	+2.1	0.012
20	DNA-directed RNA polymerase subunit alpha [*Enterobacter* sp. 638]	36 kDa	gi|145320236	12	+2.1	0.01
21	Outer membrane protein (porin) [*Enterobacter cloacae* NCTC 9394]	39 kDa	gi|295095792	5	+2	0.004

Table 6.2 Identifications of proteins highlighted as displaying differential expression between isolates TGC-S and TGC-R.

Spot no.	Protein ID	Mol wt.	Gi number	No. unique peptides matched	Expression *vs.* TGC-RΔacrB	*p* value (ANOVA)
1	Outer membrane protein (porin) [*Enterobacter cloacae* NCTC 9394]	38 kDa	gi|295095792	6	+3.4	$8.7e^{-4}$
2	Succinate dehydrogenase, flavoprotein subunit, E. coli/mitochondrial subgroup [*Enterobacter cloacae* NCTC 9394]	64 kDa	gi|295096570	11	+2.1	0.02
3	L-seryl-tRNA(Sec) selenium transferase [*Enterobacter* sp. 638]	51 kDa	gi|145316683	3	+2	$1.9e^{-4}$
4	LSU ribosomal protein L12P [*Enterobacter cloacae* NCTC 9394]	12 kDa	gi|295095128	5	+2	0.012
5	Outer membrane protein II [*Enterobacter aerogenes*]	26 kDa	gi|148368	6	-4.5	0.002
6	Outer membrane protein II [*Enterobacter aerogenes*]	26 kDa	gi|148368	5	-4.1	0.001
7	Pyruvate dehydrogenase [*Enterobacter cloacae* ATCC 13047]	62 kDa	gi|295057584	6	-3	0.004
8	DNA-binding ferritin-like protein (oxidative damage protectant) [*Enterobacter cloacae* NCTC 9394]	19 kDa	gi|295096504	9	-2.9	0.003
9	Cell division topological specificity factor MinE [*Enterobacter* sp. 638]	10 kDa	gi|145318895	2	-2.8	0.002
10	Pyrroline-5-carboxylate reductase [*Enterobacter cloacae* ATCC 13047]	28 kDa	gi|295055967	3	-2.7	0.013
11	Ribosomal protein L5 [*Enterobacter cancerogenus* ATCC 35316]	20 kDa	gi|261342778	4	-2.6	$5e^{-4}$
12	Ribosomal subunit interface protein [*Enterobacter cancerogenus* ATCC 35316]	13 kDa	gi|288315682	3	-2.5	0.005
13	Conserved hypothetical protein TIGR00255 [*Enterobacter cloacae* NCTC 9394]	33 kDa	gi|295095229	8	-2.4	0.038
14	SCP-2 sterol transfer family protein [*Enterobacter cancerogenus* ATCC 35316]	20 kDa	gi|288315250	7	-2.4	0.022
15	LuxS protein involved in autoinducer AI2 synthesis [*Enterobacter cloacae* NCTC 9394]	18 kDa	gi|295097274	3	-2.3	$6.7e^{-4}$
16	Isocitrate dehydrogenase [*Enterobacter cloacae* ATCC 13047]	46 kDa	gi|295057303	10	-2.2	0.01
17	Phosphopentomutase [*Enterobacter cloacae* NCTC 9394]	44 kDa	gi|295098516	9	-2.1	0.05
18	Acid-induced glycyl radical enzyme [*Enterobacter cloacae* NCTC 9394]	14 kDa	gi|295097890	4	-2.1	0.02
19	Dihydrodipicolinate synthase [*Enterobacter* sp. 638]	31 kDa	gi|145319491	5	-2.1	0.01
20	Maltooligosaccharide-binding protein [*Enterobacter cloacae* NCTC 9394]	44 kDa	gi|295095401	11	-2	0.016
21	Enolase [*Enterobacter cloacae* NCTC 9394]	46 kDa	gi|295097345	11	-2	0.008
22	Ribose-phosphate pyrophosphokinase [*Enterobacter cancerogenus* ATCC 35316]	34 kDa	gi|261340155	15	-2	0.006
23	Ribose-phosphate pyrophosphokinase [*Enterobacter cancerogenus* ATCC 35316]	34 kDa	gi|261340155	8	-2	0.013

Table 6.3 Identifications of proteins highlighted as displaying differential expression between isolates TGC-R and TGC-R*ΔacrB*.

6.3 Changes in protein expression that appear to associate with *acrB* upregulation

The eight proteins in this group were found to have expression patterns which mimicked that of the AcrB protein, some were positively associated *i.e.* when the efflux pump was upregulated, these proteins increased in expression, and some were negatively associated *i.e.* when the pump was upregulated, the expression of these proteins was reduced. The following two proteins displayed a positive association with AcrB, while six others displayed negative associations. All proteins in this associated group are listed in Table 6.4.

6.3.1 Proteins displaying a positive association

The outer membrane protein (spot 21; Table 6.2 and spot 1; Table 6.3) was identified as OmpD (or NmpC) by BLASTp (E = 0). Expression of OmpD was increased in TGC-R 2-fold and reduced by 3.4-fold in TGC-RΔ*acrB*. It is differentially regulated in both comparisons of the *E. cloacae* isolates and is the first of two proteins which show an increase in expression that correlates with the increased expression of the AcrB efflux pump protein. When comparing TGC-R/ TGC-RΔ*acrB*, OmpD expression drops 3.4-fold when AcrB is not expressed (in tigecycline-susceptible TGC-RΔ*acrB*).

Expression of SdhA (succinate dehydrogenase/ SDH) flavoprotein subunit; spot 17; Table 6.2 and spot 2; Table 6.3) was similarly increased in TGC-R *vs.* TGC-S by 2.3-fold and reduced by 2.1-fold in TGC-R/Δ*acrB*.

	Protein Identification
Positive association	OmpD/NmpC
	Succinate dehydrogenase flavoprotein subunit (SdhA)
Negative association	Enolase
	Glycyl radical cofactor (GrcA)
	Isocitrate dehydrogenase
	Pyruvate dehydrogenase
	Ribosomal subunit interface protein (RaiA)
	DNA protection during starvation protein (Dps)

Table 6.4 Proteins displaying expression patterns which were associated positively or negatively with efflux activity.

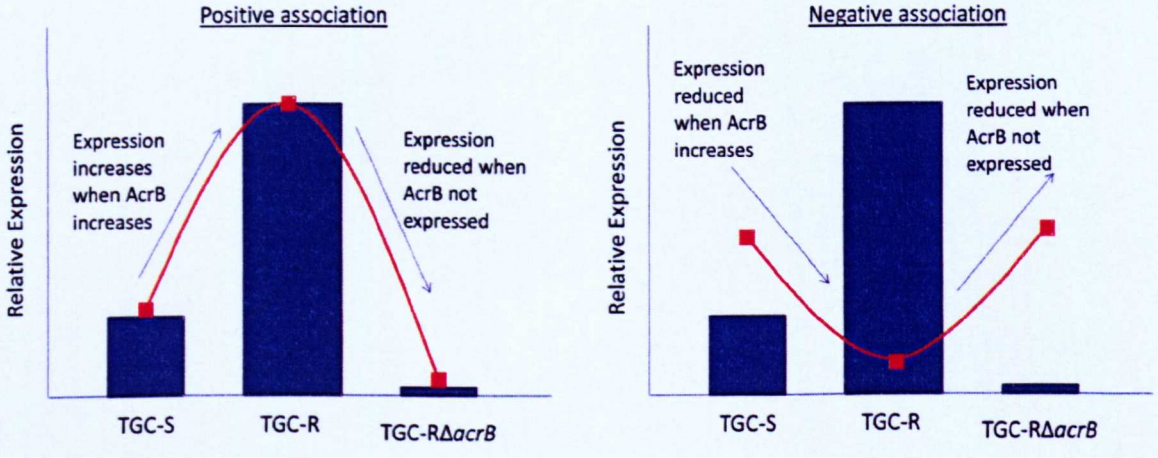

Figure 6.6. Positive and negative associations that many of the identified proteins had with AcrB observed in the three isolates tested.

6.3.2 Proteins displaying a negative association with *acrB* upregulation

The following proteins showed expression patterns that associated with the inverse of AcrABC pump expression (designated as negative association). Spots 12, 15 (Table 6.2) and spot 21 (Table 6.3) were all identified as enolase, spots 12 and 15 showed reduced expression in TGC-R by 2.1- and 2-fold respectively, while spot 21 displayed increasing expression in TGC-R/Δ*acrB* by 2-fold. Enolase converts 2-phosphoglycerate into phosphoenolpyruvate, an essential step in glycolysis and is also a component of the RNA degradosome complex which processes and decays mRNA, although its exact role in the complex remains to be determined (Carpousis 2007).

The acid-induced glycyl radical enzyme, represented by three spots of interest (spots 1, 4 and 6; Table 6.2) showing reduced expression in TGC-R by 4.6-, 2.9- and 2.5-fold respectively. This same protein displays increased expression in TGC-R/Δ*acrB* by 2.1-fold (spot 18; Table 6.3). Following BLASTp analysis, the protein showed high similarity with glycyl radical cofactor (GrcA, E value = $2e^{-87}$). GrcA is a homologue of YfiD of *E. coli* and functions to reconstitute the glycyl radical domain of pyruvate-formate lyase, which requires a glycyl radical active site under oxidative stress conditions (Wagner *et al.* 2001). This serves as a stress defence protein against oxidative environments and is essential for anaerobic growth.

Isocitrate dehydrogenase (spot 11; Table 6.2 and spot 16; Table 6.3) displayed reduced expression of 2.2-fold in TGC-R and an increase of 2.2-fold in TGC-R/Δ*acrB*. Isocitrate dehydrogenase catalyses the oxidative decarboxylation of isocitrate, producing α-ketoglutarate and CO_2 while converting NAD^+ to NADH. Pyruvate dehydrogenase (spot 9; Table 6.2 and spot 7; Table 6.3) showed a reduction in expression of 2.3-fold in TGC-R and an increase of 3-fold in TGC-R/Δ*acrB*. Pyruvate dehydrogenase catalyzes oxidative decarboxylation of pyruvate to form acetyl-CoA, both pyruvate and isocitrate dehydrogenases are vital enzymes in the TCA cycle and energy generation.

Spot 10 (Table 6.2) and spot 12 (Table 6.3) were identified as ribosomal subunit interface protein or RaiA, the expression of which was reduced 2.3-fold in TGC-R and increased 2.5-fold in TGC-R/Δ*acrB*. RaiA responds to stress by binding to the ribosome and inhibiting translation,

although its main activity is to reduce translation errors in protein biosynthesis (Agafonov & Spirin, 2004).

DNA-binding ferritin-like protein (spots 2 and 3; Table 6.2 and spot 8; Table 6.3) showed reduced expression in TGC-R of 4.4- and 2.9-fold respectively and increased expression of 2.9-fold in TGC-R/Δ*acrB*. They were later identified as DNA protection during starvation protein (Dps) by BLASTp (E value = $2e^{-92}$). Dps binds DNA non-specifically and condenses it to offer protection from a variety of damaging agents (radiation, thermal shock and pH stress) and as part of this protection, it can sequester and recycle Fe^{2+} ions to prevent them forming reactive oxygen species (Calhoun & Kwon, 2011).

6.4 Changes in expression with potential implications for virulence

Some of the differentially expressed proteins identified between the two comparisons (TGC-S *vs.* TGC-R and TGC-R *vs.* TGC-RΔ*acrB*) had the potential to alter the virulence of *E. cloacae* isolates. These include spots 5 and 6 (Table 6.3), which were both returned as OmpA protein by BLASTp analysis (E value = $7e^{-136}$) and showed an increased in expression of 4.5- and 4.1-fold respectively.

The LuxS protein (spot 15; Table 6.3) expression increased 2.3-fold in TGC-RΔ*acrB* and is part of the synthetic pathway that produces autoinducer-2 (AI-2), a molecule used for Quorum Sensing (QS) in many species of pathogenic bacteria (Rezzonico & Duffy, 2008).

The outer membrane protein (porin) was identified as OmpD by BLASTp and was increased 2-fold in TGC-R (spot 21; Table 6.2). OmpD serves a variety of functions but is frequently associated with protection, from heat and oxidative stresses for example.

6.5 Other observed protein differences

6.5.1 Differences arising between TGC-S and TGC-R

In the comparison of TGC-S *vs.* TGC-R, the expression of 11 proteins was increased in TGC-S and six of these were found to have an association with AcrB. The remaining five include: superoxide dismutase (SOD) (Spot 5; Table 6.2), which showed a 2.8-fold expression increase in TGC-S and is

involved in oxidative stress defence. Septum site-determining protein MinD (spot 7; Table 6.2) showed a 2.4-fold expression increase and is involved in regulation of MinC which inhibits septum formation at the cell poles during division (Lutkenhaus, 2007). The aldo/keto reductase (spot 8; Table 6.3) also showed a 2.4-fold expression increase and returned as the dkgA gene product via BLASTp analysis (E = 0) which is involved in fermentation in *E. coli* (Miller *et al.* 2009). The hypothetical protein ENTCAN_05473 (spot 13; Table 6.2) showed a 2.1-fold increase in expression and returned as pyruvate formate lyase via BLASTp analysis (E = 0) which catalyses the cleavage of pyruvate to formate under anaerobiosis (Buckel & Golding, 2006). The predicted periplasmic/secreted lipoprotein (spot 14; Table 6.2) also showed a 2.1-fold increase in expression and returned as OsmY via BLASTp analysis ($E = 4e^{-137}$) which is involved in protection from hyperosmotic environments.

In this same comparison, six proteins were increased in TGC-R and two of these were associated with AcrB. The remaining four include: Pyruvate ferredoxin/flavodoxin oxidoreductase (spot 16; Table 6.2) showed a 2.4-fold expression increase and catalyses the conversion of pyruvate to acetyl-coA and CO_2, while L-proline dehydrogenase (spot 18; Table 6.2), which participates in proline and arginine metabolism showed an expression increase of 2.2-fold. The unnamed protein product (spot 19; Table 6.2) showed an increase in expression of 2.1-fold and returned as Succinate dehydrogenase subunit B via BLASTp analysis ($E = 3e^{-176}$). RNA polymerase subunit alpha (spot 21; Table 6.2) expression also increased 2.1-fold and has many functions as part of the RNA polymerase complex, including recognition of transcription initiation sites and ensuring complex stability (Rippa *et al.* 2010).

6.5.2 Differences arising between TGC-R and TGC-RΔ*acrB*

In the comparison of TGC-R *vs.* TGC-RΔ*acrB*, the expression of 4 proteins was increased in TGC-R, two of which were found to have an association with AcrB. The remaining two were: L-seryl-tRNA selenium transferase (Spot 3; Table 6.3), required for the synthesis of selenoproteins and ribosomal protein L12 (spot 4; Table 6.3), which both showed a 2-fold expression increase.

In this same comparison, 17 proteins were increased in TGC-RΔ*acrB* and six of these were associated with AcrB and two (OmpA influxes) were mentioned previously in section 6.4. The remaining nine include: MinE (spot 9; Table 6.3), which showed a 2.8-fold expression increase and is a regulator of MinC and MinD activity in the inhibition of septum formation during cell division (Lutkenhaus 2007). Pyrroline-5-carboxylate reductase (spot 10; Table 6.3) showed a 2.7-fold increase in expression and is involved in L-proline biosynthesis and ribosomal protein L5 (spot 11; Table 6.3) showed a 2.6-fold increase in expression. Hypothetical protein TIGR00255 (spot 13; Table 6.3) expression increased 2.4-fold and returned as a potassium-transporting ATPase via BLASTp analysis (E = 0). SCP-2 sterol transfer protein (spot 14; Table 6.3) expression also increased 2.4-fold and returned as Yhbt via BLASTp analysis ($E = 7e^{-118}$). Phosphopentomutase (spot 17; Table 6.3) is involved in nucleic acid metabolism and its expression increased 2.1-fold. Dihydropicolinate synthase (spot 19; Table 6.3) expression increased 2.1-fold and is involved in the biosynthesis of lysine. Maltooligosaccharide-binding protein (spot 20; Table 6.3) expression increased 2-fold and functions to transport maltose across the membrane. Both spots representing ribose-phosphate pyrophosphokinase (spots 22 and 23; Table 6.3) showed a 2-fold increase in expression, also known as Prs, it is involved in the purine biosynthetic pathway.

6.6 Chapter Summary

This study has highlighted the impact that altered efflux pump expression can have on a diverse range of cellular processes in *E. cloacae*, including: changes in stress-defence proteins, changes in the levels of metabolic proteins and changes in cellular division proteins. Some of the proteins identified display a repeated pattern of expression which may be associated (either positively or negatively) with the expression of the AcrB efflux pump protein (and therefore the active pump AcrABC). These include OmpD, a porin implicated in stress resistance and SdhA or succinate dehydrogenase subunit A. Nouwen *et al.* (Nouwen *et al.* 2001) showed that a decrease in succinate dehydrogenase caused a decrease in the proton motive force (PMF) in inner membrane vesicles and as AcrABC efflux activity is driven by energy from the PMF (Martins *et al.* 2009), succinate

dehydrogenase could be (at least indirectly), supplying the energy for efflux activity. This would explain the increased expression of SdhA as a way to keep up with increased energy demand caused by increased efflux activity.

Proteins displaying a 'negative' association with AdeB include the reduced expression of enolase, which is essential in the RNA degradation complex and could suggest a decrease in mRNA processing. Although, enolase has also been implicated in cell adherence and attachment, through its fibrinogen-binding activity in pathogenic streptococci (Pancholi & Fischetti 1998) and Gram-negative species (Sha *et al.* 2009). The RNA degradosome is known to bind the cell division inhibitor protein MinD (Taghbalout & Rothfield, 2007). The precise relationship between MinD and the degradosome is unclear, but this association could explain the increased expression by both enolase and Min proteins (MinE is a regulator of MinD) in both tigecycline-susceptible isolates.

It was expected that SodB, Dps and other protection proteins demonstrated greater expression in TGC-R (the resistant isolate) rather than TGC-S. However, TGC-S still has resistances to multiple antibiotics and hence, the need for this protection. These proteins are still present in TGC-R, although their expression levels may have been affected by the increase in AcrABC activity. The increase in efflux activity in TGC-R could also contribute, as there is evidence which strongly suggests that efflux pumps participate in oxidative stress defence (Jeon *et al.* 2011). As fewer chemical challenges, toxins or antibiotics can accumulate to cause cellular damage, the need for cell defence proteins would likely decrease. The changes observed in stress defence protein expression between isolates would suggest that efflux pumps may play a larger part in stress defence than was previously thought. A reduction in the requirement for stress defence proteins and additional defence provided by increased AcrABC activity would lead to a slightly reduced need for energy, which may explain the reduced expression of TCA cycle enzymes pyruvate and isocitrate dehydrogenases. Similar findings were reported by dos Santos (2010) in an investigation into efflux-mediated resistance to the antibiotic combination piperacillin/tazobactam in *Escherichia coli* (Dos Santos *et al.* 2010). Many proteins involved in stress defence and energy metabolism demonstrated reduced in expression in the antibiotic-resistant isolate, while proteins involved in anaerobiosis demonstarted increased expression.

Other highlighted proteins have the potential to exacerbate the pathogenesis of this organism *e.g.* OmpA, OmpD and LuxS. OmpA has been shown to be an important virulence factor for closely-related *Enterobacter sakazakii,* required for bacterial attachment and invasiveness, causing persistent infection and survival in blood (Mittal *et al.* 2009). OmpA is known to be involved in bacterial attachment to host cells (Smith *et al.* 2007), as is the AcrABC efflux pump (Blair *et al.* 2009). Therefore, an increase in the expression of OmpA may have been a response to the reduced attachment capability of TGC-RΔ*acrB* caused by a lack of AcrABC.

Expression of LuxS was increased in TGC-RΔ*acrB*, possibly due to the absence of AcrB efflux pump protein. AI-2 may be a substrate for AcrABC, as this pump is known to extrude quorum sensing (QS) signal molecules from the cell (Yang *et al.* 2006). With no AcrABC activity, there will be less QS signal molecule released into the surroundings, which would mean a lack of QS-mediated control on cell growth. Increased expression of LuxS seen in TGC-RΔ*acrB* could be a response to try and increase extracellular levels of the QS signal molecule.

It has been previously reported that MDR *Enterobacter* spp. with increased efflux activity reduces porin expression (Masi *et al.* 2006). Here the opposite was observed; that OmpD expression was increased in TGC-R while efflux activity was increased compared with TGC-S. OmpD has been previously implicated in providing heat resistance to *E. coli*, (Ruan *et al.* 2011) antibiotic resistance *e.g.* to antimicrobial peptides in Salmonella (Pilonieta *et al.* 2009) and permeability-mediated resistance to carbapenems (Szabo *et al.* 2006). As the MICs of these compounds did not change significantly between the isolates studied in this chapter, it is likely that OmpD plays an alternative, unknown role that does not appear to participate in this mechanism of resistance.

Consequently, changes in the expression of these proteins could potentially make *E. cloacae* i) more resistant to stresses *e.g.* antibiotic-mediated killing, through increased OmpD levels, ii) improved OmpA-mediated attachment and invasion of host cells, and iii) persistence of infection via increased biofilm formation. Although changes in these specific proteins were not detected between the clinical pair of isolates (TGC-S and TGC-R), there is potential for increased virulence that could be conferred by proteins affected by expression levels of AcrABC. However, additional work is required to confirm the relationship between these proteins and the AcrABC

efflux pump, such as the generation and comparison of an OmpD knockout mutant (*e.g.* derived from TGC-R) with TGC-R.

This work is the first DIGE proteomic analysis of *E. cloacae* and has helped to characterise the *E. cloacae* proteome while highlighting some of the changes in protein expression associated with acquisition of efflux-mediated tigecycline resistance. The subtle changes between the isolates, detected here by DIGE, demonstrated the power of proteomics to detect previously unseen differences associated with antibiotic resistance, particularly between pairs of isolates, with the potential to identify markers of the resistance or cofactors involved in the mechanism itself.

7. Results
Tigecycline resistance in *Serratia marcescens*

7.1 Introduction of isolates

Serratia marcescens is an important nosocomial pathogen capable of causing infections in a broad range of sites and is also frequently associated with outbreaks, where it is a problematic and increasingly reported organism (Voelz *et al.* 2010). Treating *S. marcescens* infections can be problematic due to the inherent resistance to many antibiotics, and as with previous isolates described in this thesis, the remaining therapeutic options include only tigecycline and carbapenems. The broad substrate ranges of many efflux pumps can complicate treatment regimes and in an intrinsically drug-resistant species such as this, efflux-mediated resistance to multiple antibiotics could make it an extremely difficult organism to treat.

In this study a *S. marcescens* clinical isolate SM346 was used, with resistance to tigecycline (MIC = 16 mg/L) that was later attributed to up-regulation of the SdeXY-HasF tripartite efflux pump (Hornsey *et al.* 2010c). As this was a single clinical isolate with no comparator available, protein extracts of the *S. marcescens* type strain NCTC 10211 were used as a tigecycline-susceptible counterpart. The additional derivative mutants; tigecycline-resistant 10211-10, an efflux knockout 10211-10Δ*sdeY* and a second knockout mutant 10211-10Δ*hasF* (Hornsey *et al.* 2010c), were also compared with the aim of characterising the proteins associated with this efflux-mediated resistance mechanism.

Following the successful use of DIGE as described in chapters 5 and 6, the technique was applied to these isolates to see if any inferences into the effects of the resistance mechanism could be gleaned despite their differences. To date, there have been no proteomics studies carried out on *S. marcescens* with regards to antibiotic resistance and any information of the effects of efflux pumps on its protein complement would be valuable to further understanding this efflux-mediated resistance in this species.

7.2 Separation of protein extracts on gradients of pH 4-7

As previously in sections 5.2 and 6.2, the extracts of all isolates used in the DIGE proteomics experiment were tested for quality on gradient pH 4-7 gels before being labelled with the CyDyes. Based on the results of the 3-10 and 6-11 pH gradients used previously, the protein extracts were only separated on 4-7 gels.

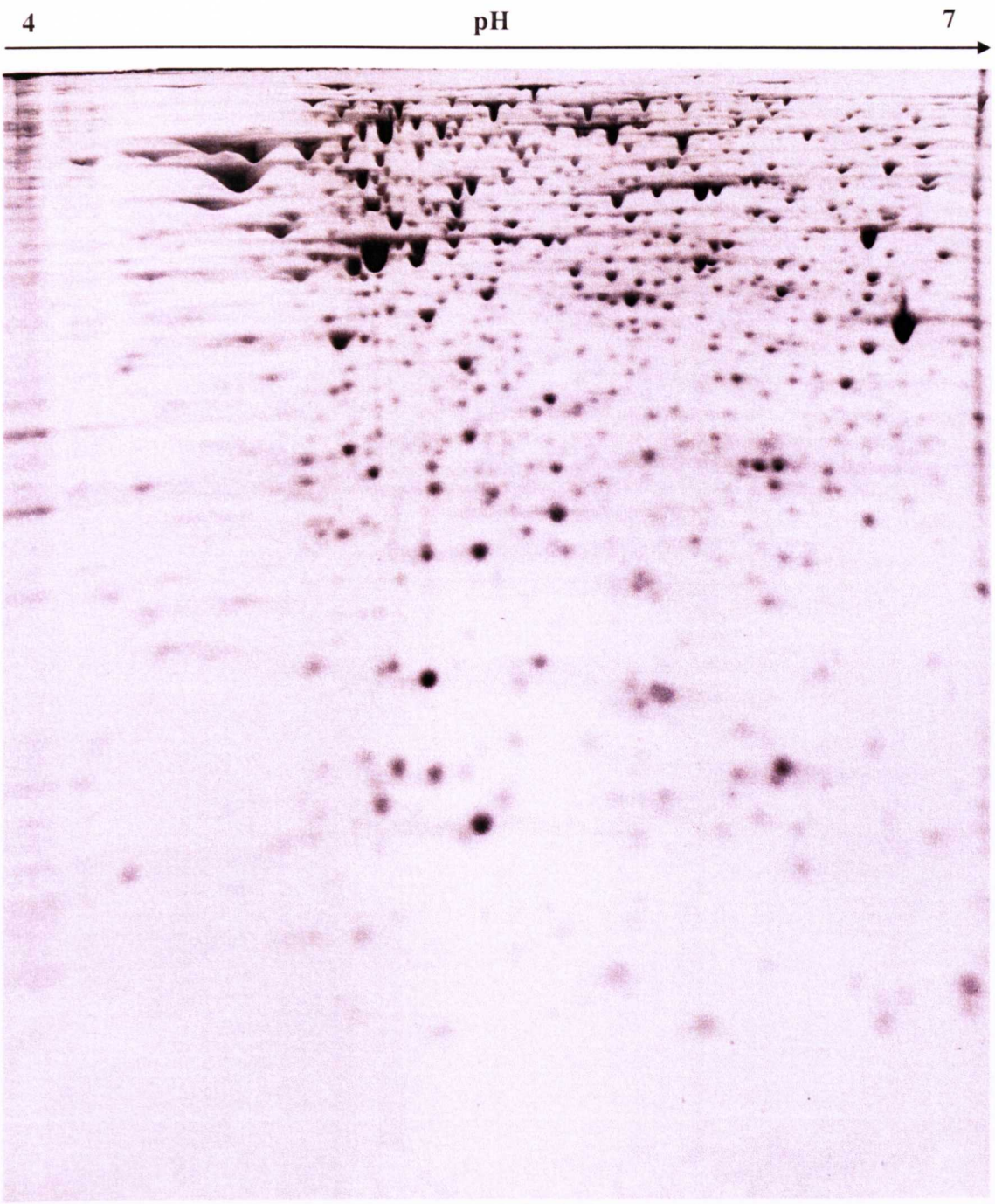

Figure 7.1 2DGE profile of proteins from isolate NCTC 10211. Total cell extract was separated using a pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

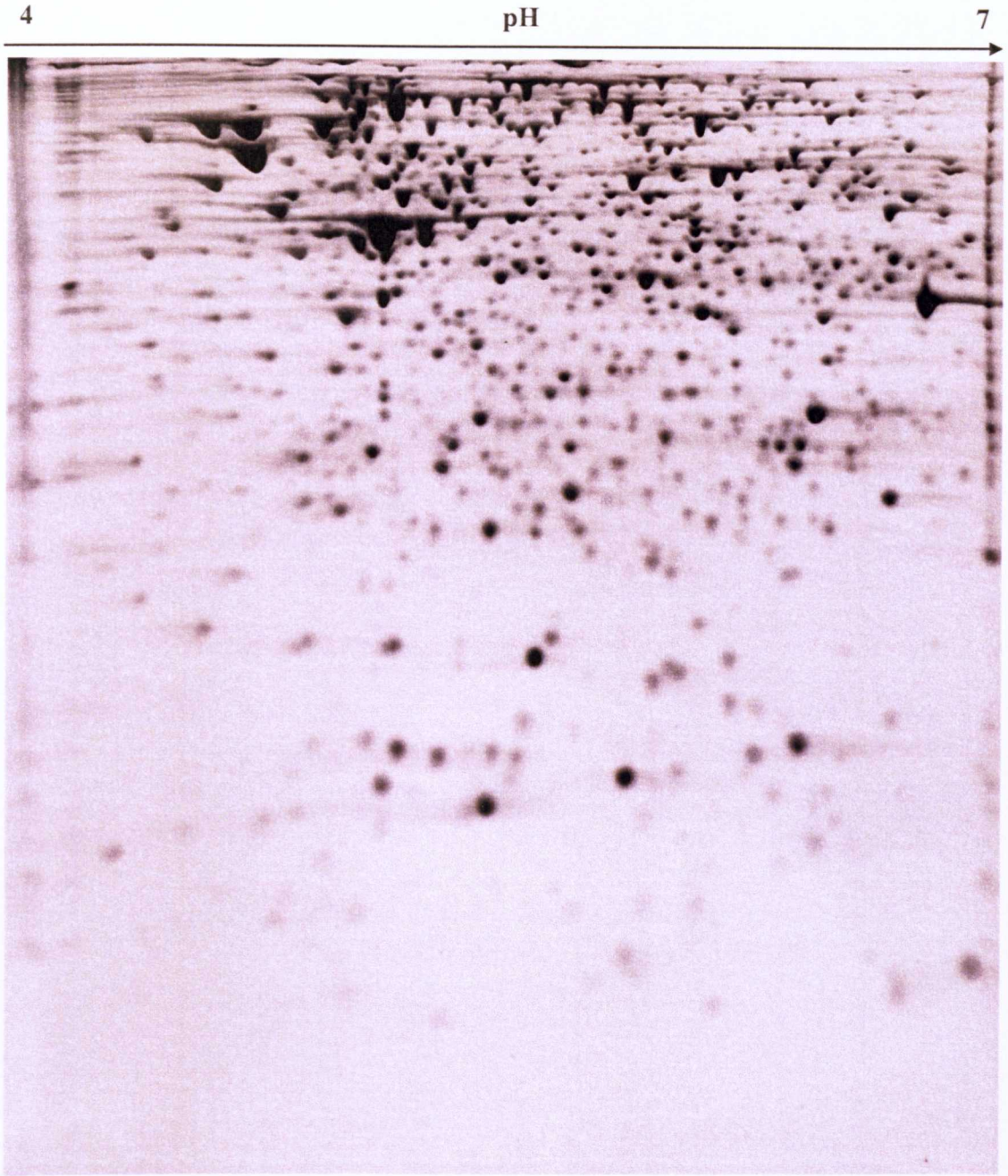

Figure 7.2 2DGE profile of proteins from isolate SM346. Total cell extract was separated using a pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

Figure 7.3 2DGE profile of proteins from laboratory mutant, isolate 10211-10. Total cell extract was separated using a pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

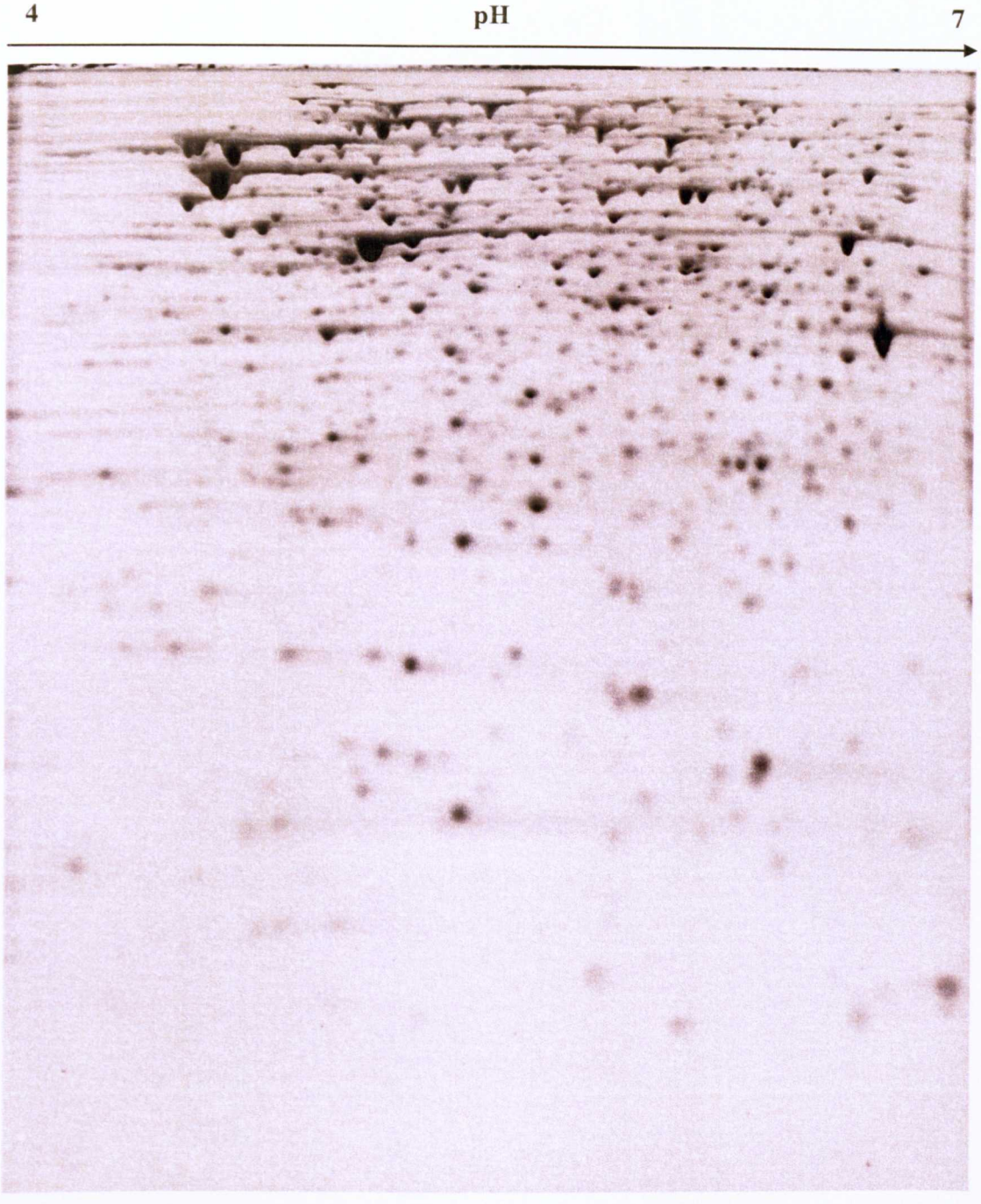

Figure 7.4 2DGE profile of proteins from knockout mutant, isolate 10211-10Δs*deY*. Total cell extract was separated using a pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

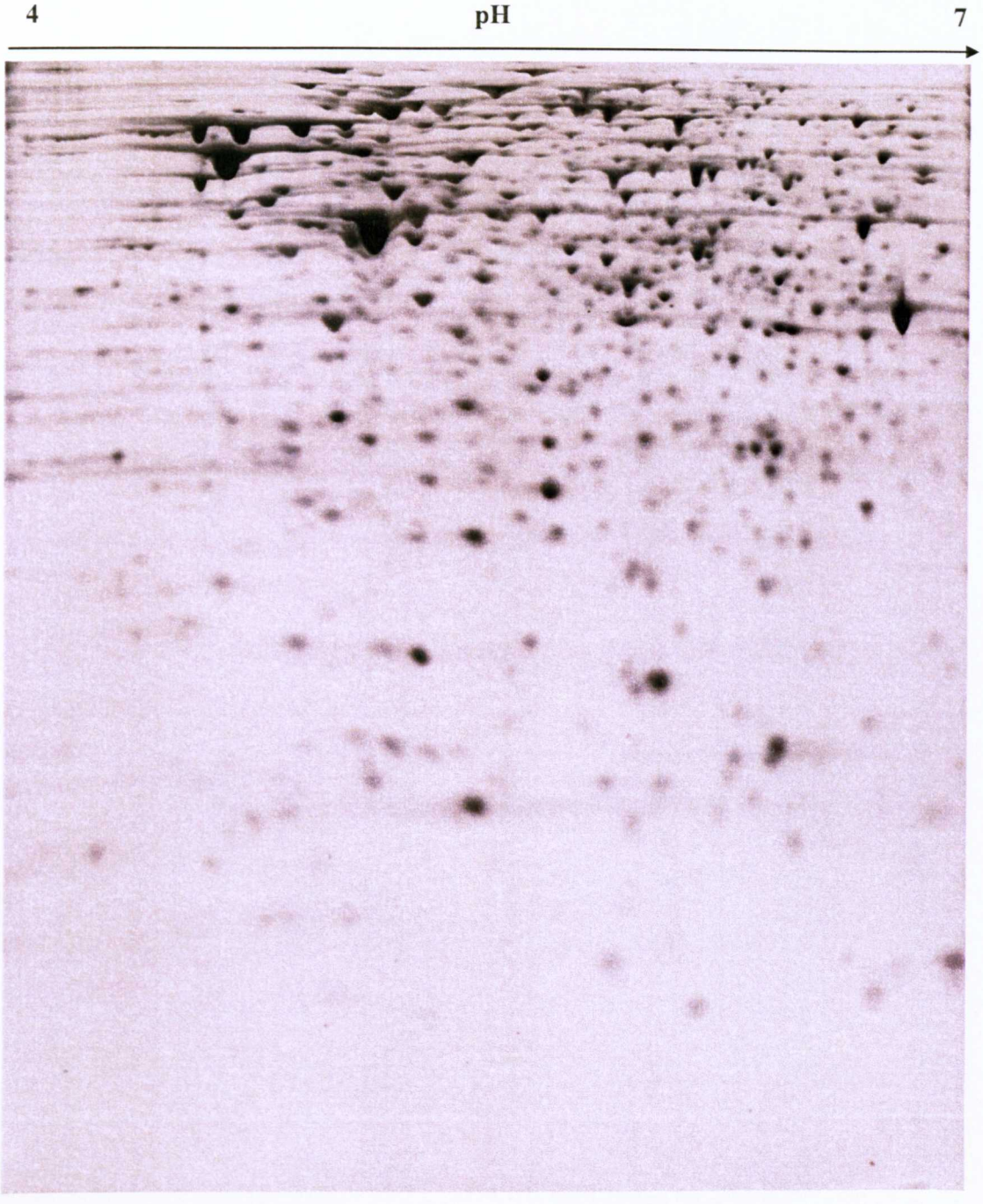

Figure 7.5 2DGE profile of proteins from knockout mutant, isolate 10211-10Δ*hasF*. Total cell extract was separated using a pH gradient of 4-7, and 12% polyacrylamide gel. The separated proteins were stained with SYPRO Ruby (Invitrogen) and visualised using an Ettan Dalt imager (GE Healthcare).

7.3 DIGE-labelled protein separations

. Approximately 720 spots were detected on the DIGE gels using the SameSpots software (Progenesis v3.03 Nonlinear Dynamics, Newcastle, UK). The isolates were labelled and grouped into gels as described in Table 7.1.

Gel no.	Cy 3	Cy 5
1	NCTC 10211 (1)	SM346 (2)
2	10211-10 (2)	ΔSdeX (1)
3	ΔHasF (1)	NCTC 10211 (2)
4	SM346 (1)	ΔHasF (2)
5	ΔSdeX (3)	10211-10 (1)
6	NCTC 10211 (3)	10211-10 (3)
7	SM346 (3)	ΔSdeX (2)
8	ΔHasF (3)	

Table 7.1 DIGE experimental setup for the *S. marcescens* isolates, numbers in brackets refer to the biological replicate of the sample

From the comparisons performed in this experimental setup, three were chosen for further analysis. Comparisons with the knockout derivatives yielded very low numbers of proteins, less than five, with differential expression. These results would have been statistically weak with such a low number of proteins and any inferences made would be purely speculative with so few identifications, therefore these few spots highlighted by SameSpots were not submitted for LC-MS/MS analysis. The three comparisons that were chosen for further analysis were as follows: (i) tigecycline-resistant clinical isolate SM346 and NCTC 10211 (ii) clinical isolate SM346 and derivative tigecycline-resistant mutant 10211-10 and (iii) resistant lab mutant 10211-10 and NCTC 10211.

7.4 Comparison of NCTC 10211 type strain with SM346 clinical isolate

7.4.1 DIGE-labelled separation of protein extracts

While these two isolates are not genetically similar as compared with the organisms in chapters 5 and 6, they were compared to test the limitations of this DIGE system, as identical pairs of bacteria are not always available when investigating unusual resistance mechanisms. The genetic dissimilarity was expected to produce many individual proteins that appeared 'unique' to either one isolate or the other. However, due to the potential problems with confirming the 'uniqueness' of proteins (see section 5.4.1), 'unique' proteins were not selected for further analysis and only the differentially expressed proteins were selected for identification.

There were 28 proteins that displayed differential regulation between NCTC 10211 and SM346, 17 of these showed increased expression in 10211 and 11 proteins showed increased expression in SM346 (Fig 7.6).

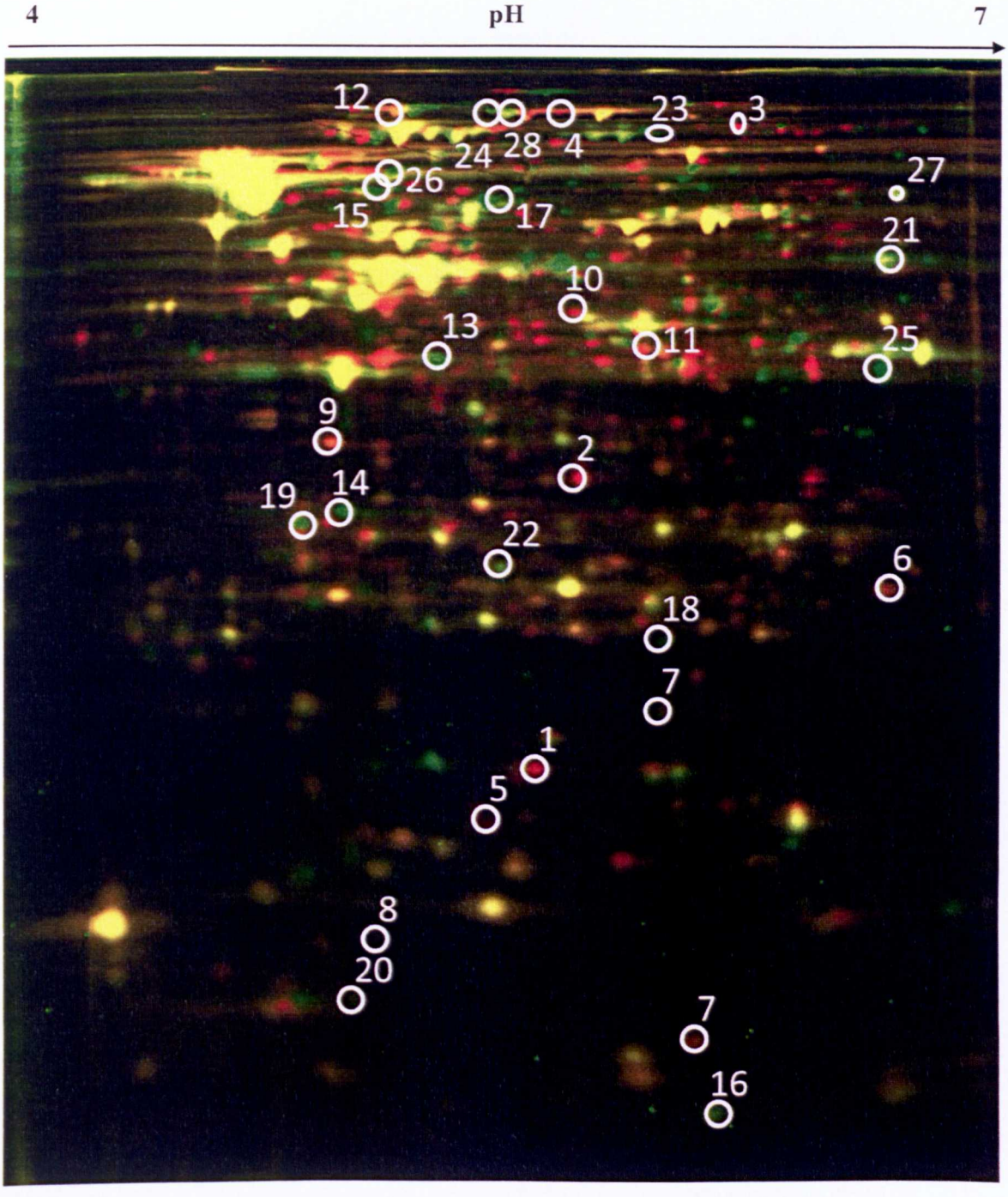

Fig. 7.6 2-D separation of DIGE-labelled proteins over a pH gradient of 4-7 and a 12% polyacrylamide gel, using extracts from SM346 (red) and NCTC 10211 (green). Numbers correspond to the proteins in tables 7.2 and 7.3.

Spot no.	Protein Identification	GI number	Mol wt.	No. of unique peptides	Fold difference in expression *vs.* NCTC 10211	*p* value (ANOVA)
1	Preprotein translocase [*Serratia odorifera* DSM 4582]	gi|291424709	100 kDa	17	9.8	$8.00e^{-3}$
2	Glycine hydroxymethyltransferase [*Serratia proteamaculans* 568]	gi|157323637	45 kDa	6	4.3	$3.00e^{-3}$
3	YceI family protein [*Serratia proteamaculans* 568]	gi|157321893	21 kDa	5	3.9	$2.00e^{-3}$
4	Single-stranded DNA-binding protein	gi|134916	19 kDa	3	3.2	$8.00e^{-3}$
5	Unnamed protein product [*Serratia marcescens* subsp. *marcescens*]	gi|2980923	57 kDa	11	3.1	$3.00e^{-5}$
6	Hypothetical protein SOD_a04090 [*Serratia odorifera* 4Rx13]	gi|270044668	85 kDa	11	2.8	$1.00e^{-2}$
7	Pyruvate dehydrogenase E1 component [*Serratia odorifera* 4Rx13]	gi|270042726	99 kDa	9	2.4	$2.00e^{-2}$
8	Aconitate hydratase 2 [*Serratia proteamaculans* 568]	gi|157324006	94 kDa	18	2.1	$4.00e^{-3}$
9	Hypothetical protein SOD_a04090 [*Serratia odorifera* 4Rx13]	gi|270044668	85 kDa	18	2.1	$6.00e^{-3}$
10	Transporter [*Serratia odorifera* 4Rx13]	gi|270041650	35 kDa	7	2.1	$7.00e^{-3}$
11	Cell division topological specificity factor MinE [*Serratia proteamaculans* 568]	gi|157322762	10 kDa	6	2	$4.00e^{-3}$

Table 7.2 Proteins displaying increased expression in SM346 in comparison with NCTC 10211 as highlighted by SameSpots software.

Spot no.	Protein Identification	GI number	Mol wt.	No. of unique peptides	Fold difference in protein expression *vs.* SM346	*p* value (ANOVA)
12	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase [*Serratia odorifera* 4Rx13]	gi|270041121	30 kDa	5	4.3	$4.00e^{-3}$
13	Extracellular solute-binding protein family 5 [*Serratia proteamaculans* 568]	gi|157322705	61 kDa	9	4.2	$2.00e^{-3}$
14	Conserved hypothetical protein [*Serratia odorifera* DSM 4582]	gi|291421655	7 kDa	3	3.1	$4.00e^{-3}$
15	ATP-dependent Clp protease, ATP-binding subunit ClpX [*Serratia proteamaculans* 568]	gi|157321103	46 kDa	5	2.7	$2.40e^{-5}$
16	Heat shock protein [*Serratia odorifera* 4Rx13]	gi|270041778	16 kDa	3	2.7	$3.00e^{-4}$
17	Phosphoserine aminotransferase [*Serratia proteamaculans* 568]	gi|157321713	40 kDa	5	2.7	$7.00e^{-3}$
18	Maf protein [*Serratia proteamaculans* 568]	gi|157324405	21 kDa	2	2.7	$1.10e^{-2}$
19	Pyruvate dehydrogenase E1 component [*Serratia odorifera* 4Rx13]	gi|270042726	99 kDa	20	2.7	$1.90e^{-2}$
20	50S ribosomal protein L24 [*Serratia odorifera* DSM 4582]	gi|291424210	11 kDa	2	2.5	$3.70e^{-2}$
21	Uracil phosphoribosyltransferase [*Serratia proteamaculans* 568]	gi|157323524	23 kDa	5	2.4	$2.00e^{-3}$
22	Peptidyl-dipeptidase Dcp [*Serratia odorifera* 4Rx13]	gi|270045217	82 kDa	10	2.2	$2.00e^{-3}$
23	Dihydrodipicolinate synthase [*Serratia marcescens*]	gi|46102532	31 kDa	10	2.2	$5.00e^{-3}$
24	Hypothetical protein SOD_h01030 [*Serratia odorifera* 4Rx13]	gi|270041572	53 kDa	8	2.2	$7.00e^{-3}$
25	Septum site-determining protein MinD [*Serratia proteamaculans* 568]	gi|157322761	30 kDa	7	2.1	$7.00e^{-3}$
26	Anaerobic ribonucleoside-triphosphate reductase large subunit [*Serratia odorifera* DSM 4582]	gi|291423457	80 kDa	5	2.1	$1.80e^{-2}$
27	RecName: Full=50S ribosomal protein L1	gi|132755	25 kDa	4	2	$5.60e^{-4}$
28	Aspartyl-tRNA synthetase [*Serratia proteamaculans* 568]	gi|157322785	66 kDa	15	2	$1.80e^{-2}$

Table 7.3 Proteins that were highlighted by SameSpots software as displaying increased expression in NCTC 10211 in comparison with SM346.

7.4.2 Proteins displaying increased expression in SM346

Many of the 11 proteins that demonstrated increased expression in SM346 as determined by SameSpots analysis, could be placed into one of three functional categories: (i) Membrane transport (section 7.4.2.1); (ii) Stress defence proteins (section 7.4.2.2) and (iii) Proteins involved in metabolism (section 7.4.2.3).

7.4.2.1 Membrane transport

The expression of preprotein translocase (spot 1; Table 7.2) increased 9.8-fold and returned as SecA by BLASTp (E = 0). SecA works with the SecYEG translocase system to export partially folded proteins across the cytoplasmic membrane. SecA has ATPase activity, thus providing energy for protein translocation (Plessis *et al.* 2011 and Sardis & Economou 2010). The Sec transport system is also required for delivering beta lactamases, such as TEM, AmpC and CTX-M enzymes, to the periplasm (Pradel *et al.* 2009) and may be important for resistance to β-lactams.

YceI family protein (spot 3; Table 7.2) expression increased by 3.9-fold, this protein is similar to YceI of *Escherichia coli*, a periplasmic protein which is induced by high pH and can bind lipids. It is thought that when YceI is activated under basic conditions, it transports molecules that will lower the pH across the membrane, such as acid-generating lipids (Stancik *et al.* 2002).

The Transporter protein (spot 10; Table 7.2) was later classified as a carbohydrate, sugar or ribose-uptake ABC transporter periplasmic-binding protein by BLASTp (E = 0) and its expression was increased 2.1-fold.

7.4.2.2 Stress Defence

Some of the proteins identified play a role in stress defence and include glycine hydroxymethyltransferase or GlyA (spot 2; Table 7.2), which displayed a 4.3-fold expression increase in SM346. This protein catalyses the interconversion of serine and glycine, hence it can also be referred to as serine hydroxymethyltransferase. GlyA is an important enzyme in one-carbon

metabolism and catalyses the conversion of tetrahydrofolate to 5,10-methylenetetrahydrofolate, an important step in both tetrahydrofolate and one-carbon metabolic pathways (Shirazi-Beechey & Knowles, 1984).

Expression of single-stranded DNA-binding protein or SSB (spot 4; Table 7.2) increased by 3.2-fold, this protein plays important roles in DNA replication, repair and recombination, which are essential for survival. During these processes, SSB binds to and protects single-stranded DNA from digestion and secondary-structure formation (Huang *et al.* 2011 and Reyes-lamothe *et al.* 2010). SSB could also confer protection against many other stresses *e.g.* osmotic stress (Weber *et al.* 2006)

Expression of an unnamed protein (spot 5; Table 7.2) was increased by 3.1-fold and was later identified as GroEL via BLASTp (E = 0). GroEL is a chaperone essential for cellular growth, which quarantines newly synthesised polypeptide chains from the cytosol. It then folds/re-folds them in the absence of similarly aggregative polypeptides, this ensures correct protein folding and prevents the formation of protein aggregates (Chaudhuri *et al.* 2009). Mutations in this gene in *E. coli* have demonstrated increased susceptibility to fluoroquinolones (Yamaguchi *et al.* 2003), therefore it may play important role in the stress response against antimicrobials in *S. marcescens*.

7.4.2.3 Metabolic processes

There were two hypothetical proteins which both returned as formate acetyltransferase or pyruvate formate lyase (Pfl) by BLASTp (E = 0) and displayed expression increases of 2.8-fold (spot 6; Table 7.2) and 2.1-fold (spot 9; Table 7.2). This protein is required for the reversible conversion of pyruvate and CoA into formate and acetyl-CoA under anaerobic respiration, as its glycyl radical is highly sensitive to oxygen attack.

Pyruvate dehydrogenase E1 component (spot 7; Table 7.2) was increased 2.4-fold, pyruvate dehydrogenase decarboxylates pyruvate to acetyl-CoA and links the pathways of glycolysis and the citric acid cycle. This protein was identified in section 6.3.2 as increased in tigecycline-susceptible isolates and reduced in -resistant isolates.

The expression of aconitate hydratase 2 (spot 8; Table 7.2) also known as aconitase B or AcnB, was increased 2.1-fold in SM346. Aconitase B catalyses the interconversion of citrate and isocitrate in the TCA cycle, it may have importance in iron regulation, growth and superoxide/radical sensitivity due to its key function and essential 4Fe-4S cluster, which would be very sensitive to changes in the level of available iron in the environment (Varghese *et al.* 2003). Other functions stem from its ability to bind mRNA and sensitivity to iron/superoxide, which allow it to switch between central metabolism and regulatory functions (Tang *et al.* 2005). AcnA was identified in section 5.5.2 as increased in tigecycline-resistant AB210-6 *vs.* AB211.

7.4.2.4 Other proteins increased in SM346

Finally, the cell division inhibitor protein MinE (spot 11; Table 7.2) was increased by 2-fold. MinE is a regulator of the MinCD proteins, together, MinCDE acts to inhibit formation of the FtsZ (Z-ring) complex, which primes the cell for division. The Min system ensures that this division occurs at mid-cell rather than at the poles (Lutkenhaus, 2007).

7.4.3 Proteins displaying increased expression in NCTC 10211

SameSpots analysis demonstrated an increase in the expression of 17 proteins in NCTC 10211 compared with SM346. The identified proteins were placed into the following functional categories: i) Metabolic processes (section 7.4.3.1), ii) Stress defence (section 7.4.3.2) and iii) Cell division (section 7.4.3.3).

7.4.3.1 Metabolic processes

2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (DapD) (spot 12; Table 7.3) expression increased 4.3-fold and dihydrodipicolinate synthetase (DapA) (Spot 23; Table 7.3) expression also increased, 2.2-fold in NCTC 1021. These two proteins are involved in the essential diaminopimelic acid (DAP) biosynthetic pathway of lysine and its precursor, *meso*-

diaminopimelate, a component of the cell wall peptidoglycan and have been suggested as promising therapeutic targets (Schnell *et al.* 2012).

Pyruvate dehydrogenase E1 component (Spot 19; Table 7.3) expression was increased 2.7-fold as was uracil phosphoribosyltransferase (Upp) (Spot 21; Table 7.3), expression of which was increased 2.4-fold in NCTC 10211. Upp reversibly converts uracil and ribose triphosphate into uridine monophosphate and diphosphate as part of nucleic acid metabolism.

Hypothetical protein SOD_h01030 (Spot 24; Table 7.3) expression increased 2.2-fold and returned as the pkcA gene product, phosphoenolpyruvate carboxykinase via BLASTp analysis (E = 0) which generates pyruvate in glycolysis.

Aspartyl-tRNA synthetase or AspS (Spot 28; Table 7.3) expression increased 2-fold, and catalyses the attachment of aspartate to its corresponding tRNA for delivery to the ribosome. tRNA synthetases can have alternative functions such as modification of cell peptidoglycan (Villet *et al.* 2007).

7.4.3.2 Stress defence

The conserved hypothetical protein (spot 14; Table 7.3) was identified as the cold-shock-like protein CspC and demonstrated increased expression of 3.1-fold. CspC also possesses nucleic acid-binding sites involved in regulation, it has been suggested that CspC stabilises *rpoS* transcripts, which encode the alternative sigma factor RpoS and is a major regulator of the general stress response (Cohen-or *et al.* 2010).

The expression of the ATP-dependent Clp protease subunit; ATP-binding subunit ClpX (spot 15; Table 7.3) was increased 2.7-fold in the NCTC strain. ClpX is the chaperone unit of a two-component protease, recognising and unfolding proteins for proteolysis by ClpP, a serine protease. Recently ClpX was proposed to possess diverse functions, including modulation of cell division via FtsZ degradation (Camberg *et al.* 2011).

The heat shock protein (spot 16; Table 7.3) was identified as IbpB by BLASTp ($E = 5e^{-99}$) and showed a 2.7-fold increase in expression. IbpB has been demonstrated to increase in isolates of

E. coli displaying increased propensity for biofilm formation, although its exact role in biofilm growth is unknown (Kuczynska-Wisnik *et al.* 2010).

7.4.3.3 Cell division

Phosphoserine aminotransferase (spot 17; Table 7.3) showed an increase in expression of 2.7-fold. An alternative role for SerC has been proposed by Mouslim *et al.*, suggesting that SerC is a cell division antagonist, a property independent of its phosphoserine catalytic activities (Mouslim *et al.* 2000).

The Maf protein (spot 18; Table 7.3) increased in expression by 2.7-fold in NCTC 10211. The major role of Maf is in controlling the cellular division process through inhibition of the division septum, Maf also has nucleic acid-binding activity (Hamoen, 2011)

Septum site-determining protein MinD (Spot 25; Table 7.3) expression increased 2.1-fold. Together, MinCDE acts to inhibit formation of the FtsZ (Z-ring) complex, which primes the cell for division. The Min system ensures that this division occurs at mid-cell rather than at the poles (Lutkenhaus, 2007). This expression difference in MinD was expected, as MinE (negative regulator of MinD) was increased in SM346.

7.4.3.4 Other proteins increased in NCTC 10211

Extracellular solute-binding protein family 5 (spot 13; Table 7.3) was identified by BLASTp as belonging to the OppA family (oligopeptide permease) (E value = 0). It has been demonstrated that OppA is important for cytoadhesion in *Mycoplasma hominis* (Hopfe *et al.* 2011), OppA is located in the periplasm and binds oligopeptides for transport across the membrane. Peptidyl-dipeptidase (Dcp) (Spot 22; Table 2) also increased expression 2.2-fold, it is an exopeptidase which removes dipeptides from the C-terminal of its substrates and displays structural and functional similarities to OppA (Conlin *et al.* 1995).

Lastly, there were two ribosomal protein identified in 10211, 50S ribosomal protein L24 (Spot 20; Table 7.3) and 50S ribosomal protein L1 (Spot 27; Table 7.3) which showed expression

increases of 2.5- and 2-fold, respectively. The anaerobic ribonucleoside-triphosphate reductase large subunit (Spot 28; Table 7.3) showed an increase in expression of 2.1-fold, although it returned as transcription anti-termination factor NusG via BLASTp ($E = 1e^{-128}$). This protein modulates transcription elongation and termination (Saxena & Gowrishankar, 2011).

7.5 Comparison of SM346 and laboratory mutant 10211-10

These two tigecycline-resistant isolates were compared as they possessed the same resistance mechanism, but acquired it under different circumstances *e.g. in vivo* and *in vitro* acquisition. The aim of this comparison was to compare the results with those from sections 7.4 and 7.6 to try and determine which changes are associated with upregulated efflux and which are likely to be unrelated differences between the clinical isolate and the 10211 isolates. For example, any consistent changes between the resistant isolates (SM346 and 10211-10) and susceptible NCTC 10211 may be associated with efflux upregulation. While similar changes from the comparisons of SM346 and 10211 isolates (SM346 *vs.* 10211-10 and SM346 *vs.* NCTC 10211) may be put down to genetic dissimilarity between SM346 and the 10211 isolates.

There were 13 proteins that displayed differential regulation between 10211-10 and SM346 (Fig. 7.7), six of these showed increased expression in 10211 and seven showed increased expression in SM346.

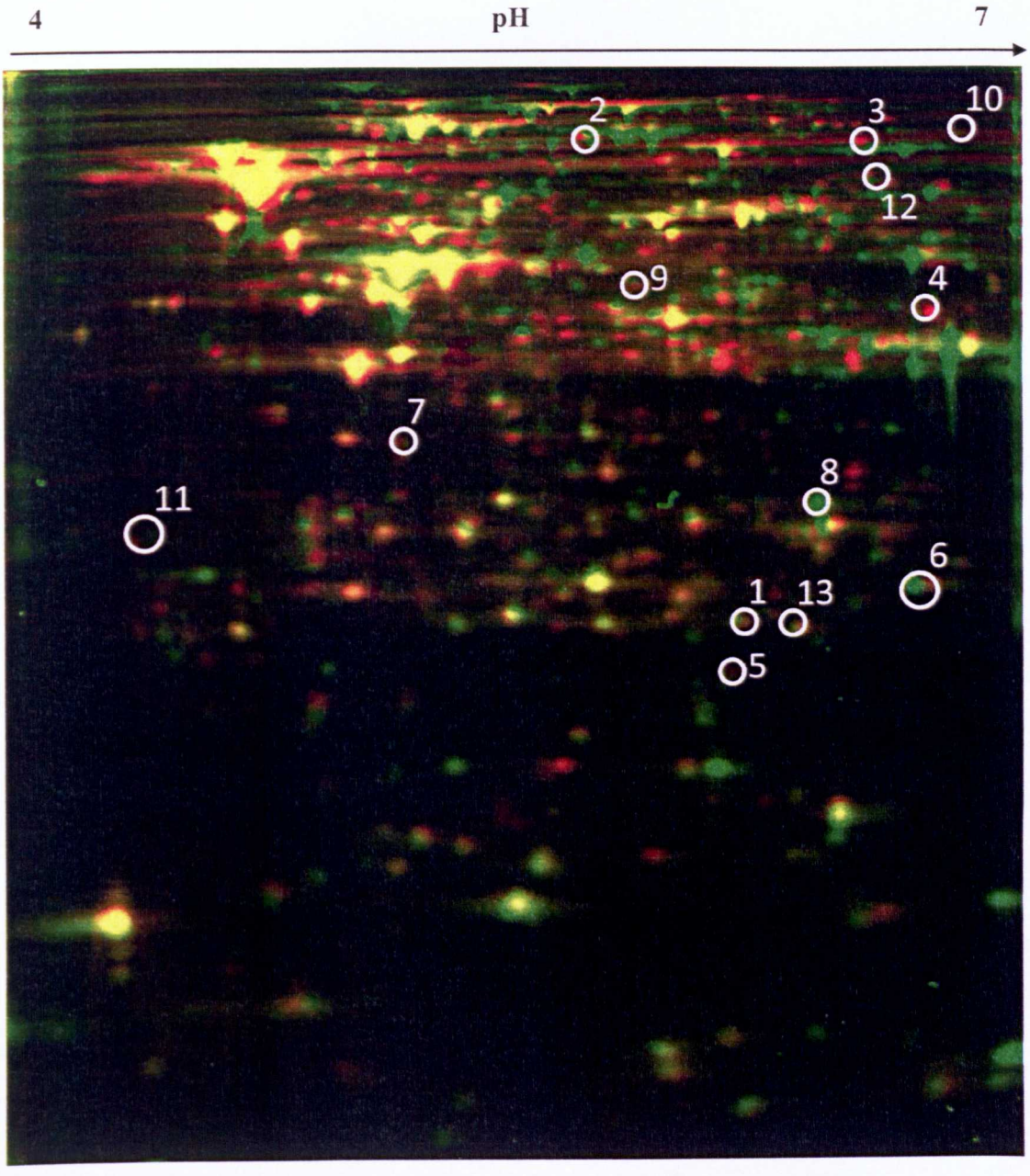

Fig. 7.7 2-D separation of DIGE-labelled proteins over a pH gradient of 4-7 and a 12% polyacrylamide gel, using extracts from SM346 (red) and 10211-10 (green). Numbers correspond to the proteins in table 7.4.

Spot no.	Protein Identification	GI number	Mol. wt.	No. of unique peptides	*p* value (ANOVA)	Fold expression difference *vs.* 10211-10
1	Transcriptional regulatory protein [*Serratia odorifera* 4Rx13]	gi|270265174	38 kDa	**3**	**$2.80e^{-2}$**	**4.5**
2	Branched-chain amino acid aminotransferase [*Serratia proteamaculans* 568]	gi|157372990	34 kDa	**4**	**$4.00e^{-3}$**	**2.7**
3	Succinate dehydrogenase [*Serratia odorifera* DSM 4582]	gi|293397236	64 kDa	**7**	**$3.10e^{-2}$**	**2.4**
4	UTP-glucose-1-phosphate uridylyltransferase [*Serratia* sp. AS12]	gi|333927590	33 kDa	**3**	**$6.00e^{-3}$**	**2.1**
5	fructose-1,6-bisphosphatase [*Serratia proteamaculans* 568]	gi|157368711	37 kDa	**9**	**$9.00e^{-3}$**	**2.1**
6	Peptidase PmbA [*Serratia proteamaculans* 568]	gi|157372617	48 kDa	**4**	**$2.70e^{-2}$**	**2.1**
7	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase [*Serratia proteamaculans* 568]	gi|157372242	93 kDa	**15**	**$5.00e^{-3}$**	**2**
8	Azoreductase [*Serratia proteamaculans* 568]	gi|157370836	21 kDa	**2**	**$6.00e^{-3}$**	**-6.6**
9	Ferritin Dps family protein [*Serratia proteamaculans* 568]	gi|157370928	18 kDa	**4**	**$2.00e^{-3}$**	**-3.1**
10	Protein chain elongation factor EF-Tu [*Serratia symbiotica* str. *Tucson*]	gi|320540641	43 kDa	**2**	**$7.00e^{-3}$**	**-2.9**
11	DNA-binding transcriptional regulator PhoP [*Serratia proteamaculans* 568]	gi|157370255	25 kDa	**8**	**$7.30e^{-4}$**	**-2.8**
12	Protein ElaB [*Serratia odorifera* 4Rx13]	gi|270263208	11 kDa	**2**	**$7.00e^{-3}$**	**-2.6**
13	Protease DO [*Serratia odorifera* DSM 4582]	gi|293392832	50 kDa	**10**	**$6.00e^{-3}$**	**-2.5**

Table 7.4 Proteins that were highlighted by SameSpots software as displaying differential expression between isolates SM346 and 10211-10.

7.5.1 Proteins displaying increased expression in SM346

7.5.1.1 Proteins involved in metabolic processes

The majority of the proteins (5/7) increased in SM346 *vs.* 10211-10 were involved in metabolism, these include branched-chain amino acid aminotransferase (Spot 2; Table 7.4) which returned as IlvE by BLASTp analysis (E = 0) and displayed a 2.7-fold increase in expression. IlvE is involved in branched-chain amino acid degradation, branched-chain fatty acid production and has shown to be important in acid tolerance in *Streptococcus mutans* (Santiago *et al.* 2012).

Succinate dehydrogenase or Sdh (Spot 3; Table 7.4) expression increased 2.4-fold and returned as SdhA, the flavoprotein subunit, via BLASTp analysis (E = 0). SdhA was described previously in Chapter 6 and has been shown to be associated with AcrAB expression in *E. cloacae.*

UTP-glucose-1-phosphate uridylyltransferase (Spot 4; Table 7.4) expression increased 2.1-fold and is the product of the galU gene.

Fructose-1,6-bisphosphatase or Fbp (Spot 5; Table 7.4) expression also increased 2.1-fold, fbp is involved in the pentose phosphate pathway.

Bifunctional aconitate hydratase 2, also Aconitate hydratase B or AcnB (Spot 7; Table 7.4), expression increased 2-fold in SM346 and was identified in section 7.4.2 as increased in SM346 *vs.* NCTC 10211. The protein AcnA was indentified in section 5.5.2 as increased in AB210-6 *vs.* AB211.

7.5.1.2 Other proteins increased in SM346

The remaining two proteins demonstrating increased expression in SM346 were the transcriptional regulatory protein (Spot 1; Table 7.4) which showed an expression increase of 4.5-fold in SM346, this protein returned as cytidine repressor protein (CytR) by BLASTp analysis (E = 0) and regulates genes the transport and catabolism of nucleosides. It is from this monitoring of nucleoside levels that CytR is said to have inhibitory activity on exopolysaccharide and biofilm formation, Haugo suggests CytR is a mechanism to time biofilm formation with a plentiful supply of nucleosides (Haugo & Watnick 2002).

The second protein demonstrating increased expression was Peptidase PmbA (TldD) (Spot 6; Table 7.4) which increased expression 2.1-fold. TldD is part of the TldDE proteolytic complex and modulator of DNA gyrase B (Allali *et al.* 2002).

7.5.2 Proteins displaying increased expression in 10211-10

Azoreductase (Spot 8; Table 7.4) expression increased 6.6-fold, azoreductase (AzoR in *E. coli*) cleaves azo compounds into their corresponding aromatic amines and is involved in resistance to thiol-specific stress (Liu *et al.* 2009).

Ferritin Dps family protein (Spot 9; Table 7.4) expression increased 3.1-fold, it binds and condenses DNA to protect it from a variety of stresses.

Protein chain elongation factor EF-Tu (Spot 10; Table 7.4) expression increased 2.9-fold. EF-Tu was previously described in section 5.7.1 and works to elongate polypeptide chains in protein synthesis but has a variety of other activities, including chaperoning and DNA repair.

Transcriptional regulator PhoP (Spot 11; Table 7.4) expression increased 2.8-fold in 10211-10, PhoP is part of a two-component response regulator PhoP/PhoQ, which controls magnesium homeostasis and governs the expression of critical virulence phenotypes in pathogenic bacteria.

Protein ElaB (Spot 12; Table 7.4) expression increased 2.6-fold and also known as YqjD in *E. coli*. This protein is poorly characterised, although it has an *E. coli* paralogue YqjD, which is bound to the inner membrane and can also bind to ribosomes. Overexpression of YqjD has been suggested to inhibit cell growth, possibly through inactivation of ribosomes (Yoshida *et al.* 2012).

Protease DO (Spot 13; Table 7.4) expression increased 2.5-fold and returned as a periplasmic serine protease, product of the htrA/degP gene by BLASTp (E = 0), serine proteases are known virulence factors for a variety of Gram negative pathogens.

7.6 Comparison of NCTC 10211 and tigecycline-resistant derivative 10211-10

The two isolates NCTC 10211 and the laboratory mutant 10211-10, were compared to investigate whether any increases in protein expression found in resistant 10211-10 were also found in SM346 *vs.* NCTC 10211 and therefore more likely to be associated with upregulation of SdeXYF, rather than an unrelated difference in expression between the reference and clinical isolates.

There were 18 spots highlighted by SameSpots as displaying differential expression, 15 of which showed increased expression in 10211-10 and three showed increased expression in NCTC 10211.

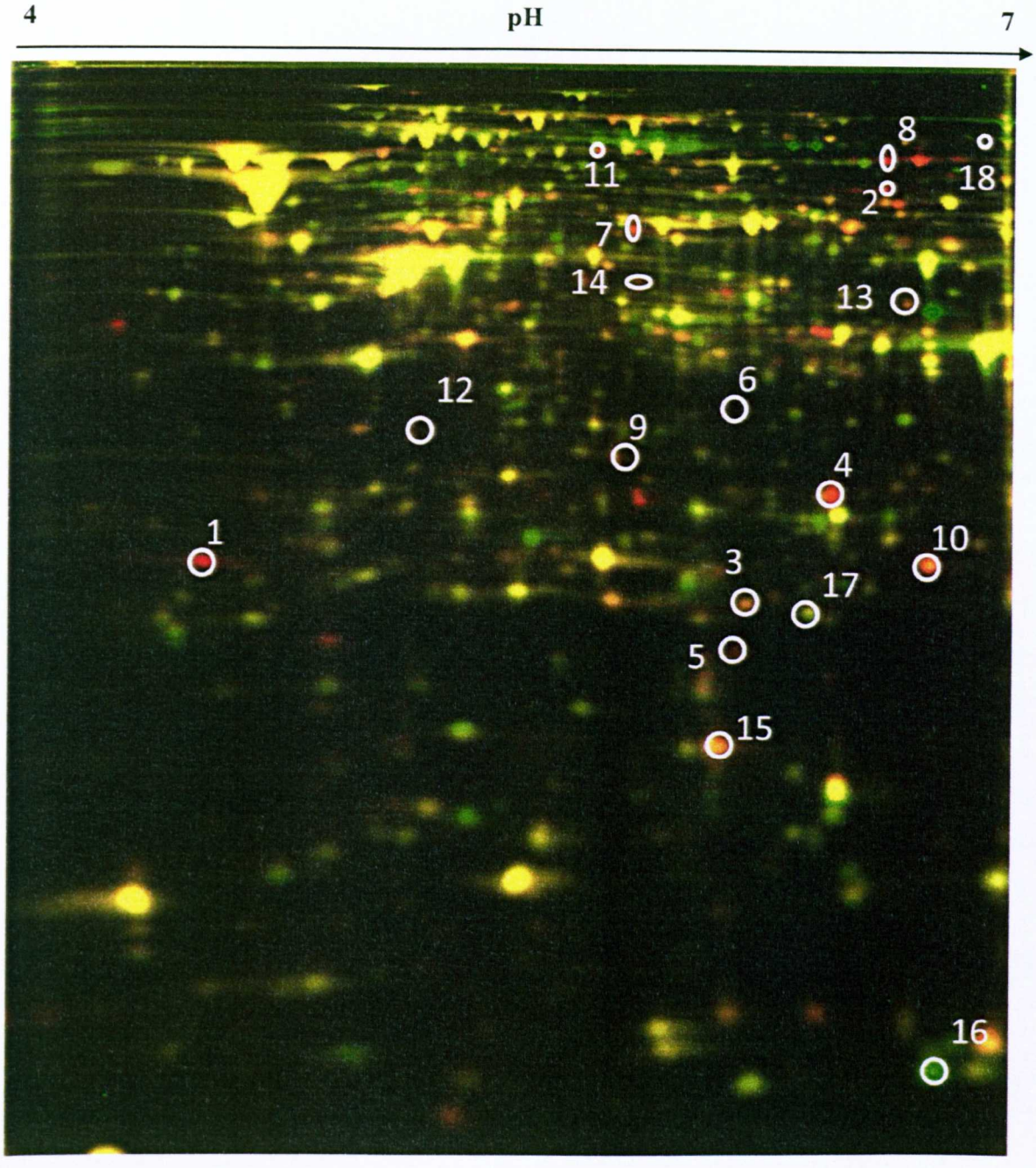

Fig. 7.8 2-D separation of DIGE-labelled proteins over a pH gradient of 4-7 and a 12% polyacrylamide gel, using extracts from NCTC 10211 (green) and 10211-10 (red). Numbers correspond to the proteins in table 7.5.

Spot No.	Protein Identification	GI number	Mol. wt.	No. unique peptides	Fold expression difference *vs.* NCTC 10211	*p* value (ANOVA)
1	Cpn60 chaperonin GroEL, large subunit of GroESL [*Serratia symbiotica* str. *Tucson*]	gi|320539693	57 kDa	2	13.4	$1.90e^{-4}$
2	Phosphoheptose isomerase [*Serratia proteamaculans* 568]	gi|157369195	21 kDa	4	5.8	$4.00e^{-3}$
3	Superoxide dismutase [*Serratia proteamaculans* 568]	gi|157368330	23 kDa	2	3.7	$3.60e^{-2}$
4	Cell division inhibitor MinD [*Serratia proteamaculans* 568]	gi|157370997	29 kDa	6	3.5	$3.00e^{-3}$
5	Hypothetical protein SOD_c05700 [*Serratia odorifera* 4Rx13]	gi|270262938	83 kDa	8	3.2	$1.10e^{-2}$
6	YceI family protein [*Serratia proteamaculans* 568]	gi|157370129	19 kDa	6	3.1	$1.10e^{-2}$
7	Short-chain dehydrogenase/reductase SDR [*Serratia odorifera* 4Rx13]	gi|270261477	26 kDa	3	2.9	$2.00e^{-2}$
8	Hypothetical protein SOD_i00600 [*Serratia odorifera* 4Rx13]	gi|270264643	21 kDa	5	2.9	$4.30e^{-2}$
9	Carboxymethylenebutenolidase [*Serratia proteamaculans* 568]	gi|157368492	30 kDa	2	2.8	$1.80e^{-2}$
10	Ferritin Dps family protein [*Serratia proteamaculans* 568]	gi|157370928	18 kDa	4	2.6	$3.50e^{-2}$
11	Carbamoyl-phosphate synthase small chain [*Serratia odorifera* 4Rx13]	gi|270263911	41 kDa	4	2.5	$1.00e^{-3}$
12	Hypothetical protein SOD_a04090 [*Serratia odorifera* 4Rx13]	gi|270261184	78 kDa	14	2.3	$2.00e^{-3}$
13	DNA-binding transcriptional regulator PhoP [*Serratia proteamaculans* 568]	gi|157370255	25 kDa	9	2.1	$3.00e^{-3}$
14	30S ribosomal protein S4 [*Serratia proteamaculans* 568]	gi|157372753	23 kDa	2	2	$1.00e^{-3}$
15	Cold-shock DNA-binding domain-containing protein [*Serratia proteamaculans* 568]	gi|157371292	7 kDa	4	2	$2.40e^{-2}$
16	Outer membrane protein TolC [*Serratia odorifera* DSM 4582]	gi|293394319	54 kDa	8	-2.6	$2.20e^{-2}$
17	Aromatic amino acid aminotransferase [*Serratia proteamaculans* 568]	gi|157372678	44 kDa	7	-2.3	$3.00e^{-3}$
18	Pyruvate dehydrogenase [*Serratia odorifera* DSM 4582]	gi|293396857	62 kDa	9	-2.2	$5.00e^{-3}$

Table 7.5 Proteins that were highlighted by SameSpots software as displaying differential expression between NCTC 10211 and 10211-10.

7.6.1 Proteins displaying expression increases in isolate 10211-10

The 15 proteins which demonstrated expression increases in 10211-10 were placed into three functional categories; i) Stress defence (section 7.6.1.1), ii) Metabolic processes (section 7.6.1.2) and iii) Potential virulence determinants (section 7.6.1.3).

7.6.1.1 Proteins involved in stress defence

GroEL, large subunit of GroESL (Spot 1; Table 7.5) expression increased 13.4-fold in 10211-10, this protein was identified and described in section 7.4.2 as increased in SM346.

Superoxide dismutase (SOD) (Spot 3; Table 7.5) expression increased 3.7-fold, SOD is an antioxidant stress defence protein, which catalyses the dismutation of superoxide, which can cause irreversible damage to nucleic acids, protein and lipids, to hydrogen peroxide.

Hypothetical protein SOD_i00600 (Spot 8; Table 7.5) expression increased 2.9-fold and returned as spermidine N1-acetyltransferase ($E = 6e^{-132}$). This protein functions to acetylate polyamines to prevent polyamine accumulation, which can be toxic, and is increased under conditions of cold shock or stress (Limsuwun & Jones 2000).

Ferritin Dps family protein (Spot 10; Table 7.5) expression increased 2.6-fold, this protein was previously identified in section 7.5 as increased in 10211-10 *vs.* SM346.

Cold-shock DNA-binding domain-containing protein or Csp (Spot 15; Table 7.5) expression increased 2-fold, Csp proteins are RNA chaperones activated by low temperatures (<15°C) which destabilise the unwanted secondary structures formed by RNA molecules (Phadtare & Severinov 2009).

7.6.1.2 Proteins involved in metabolic processes

YceI family protein (Spot 6; Table 7.5) expression increased 3.1-fold, this periplasmic protein is induced by high pH and can bind lipids. It was identified as increased in SM346 *vs.* NCTC 10211 (section 7.4.2).

Short-chain dehydrogenase/reductase SDR (Spot 7; Table 7.5) expression increased 2.9-fold and returned as 3-oxoacyl acyl carrier protein (ACP) reductase, product of the FabG gene by BLASTp analysis ($E = 4e^{-178}$). As part of the fatty acid synthase multienzyme complex, FabG catalyses an essential step in fatty acid elongation, this protein was also identified in section 5.5.1 as increased in AB211 *vs.* AB210-6.

Carboxymethylenebutenolidase (Spot 9; Table 7.5) expression increased 2.8-fold, this protein has dienelactone hydrolase activity which is involved in the degradation of chlorocatechols, intermediates in the catabolism of chlorinated aromatic compounds.

Carbamoyl-phosphate synthase small chain or CarA (Spot 11; Table 7.5) expression increased 2.5-fold, it is an essential enzyme in arginine and pyrimidine metabolism and may have a role in nitrosative stress defence in *Coxiella burnetii* (Park *et al.* 2010).

Hypothetical protein SOD_a04090 (Spot 12; Table 7.5) expression increased 2.3-fold and returned as formate acetyltransferase by BLASTp analysis (E = 0). Also known as pyruvate formate lyase, this protein was identified in section 7.4.2 as increased in SM346 *vs.* NCTC 10211.

7.6.1.3 Potential virulence determinants

There were three proteins identified with the potential to increase the virulence of *S. marcescens*, based on the current literature. The first is phosphoheptose isomerase, the *gmhA* gene product (Spot 2; Table 7.5) showed a 5.8-fold expression increase and is essential for native LPS biosynthesis in *E. coli* (Kneidinger *et al.* 2002).

Hypothetical protein SOD_c05700 (Spot 5; Table 7.5) expression increased 3.2-fold and returned as the fepA gene product, a TonB-dependant outer membrane siderophore receptor by BLASTp analysis (E = 0). FepA or ferric enterobactin protein, binds enterobactin carrying iron and transports it across the outer membrane, where it is taken across the inner membrane by FepB (Newton *et al.* 2010).

DNA-binding transcriptional regulator PhoP (Spot 13; Table 7.5) expression increased 2.1-fold. PhoP is part of a two-component response regulator PhoP/PhoQ, which controls magnesium

homeostasis and virulence phenotypes. This protein was previously identified in section 7.5.2 in 10211-10.

7.6.1.4 Other proteins increased in 10211-10

Cell division inhibitor MinD (Spot 4; Table 7.5) expression increased 3.5-fold, MinD functions to activate MinC which inhibits cell septum formation at the poles, leading to division at the correct midpoint of the cell.

7.6.2 Proteins displaying expression increases in isolate NCTC 10211

There were only three proteins in this comparison that were identified as displaying increased expression in NCTC 10211, these included the outer membrane protein TolC (Spot 16; Table 7.5) which showed an expression increase of 2.6-fold in NCTC 10211. TolC and HasF are both outer OMPs and components of tripartite efflux systems. As the SdeXY pump (and therefore HasF) expression is increased in 10211-10, a concomitant reduction in TolC was expected, as it seems logical to reduce overall energy costs and make less of the OMP not needed for drug efflux. This seems a reasonable theory as TolC can be substituted for HasF in the SdeXY efflux pump (Chen *et al.* 2003).

Aromatic amino acid aminotransferase (Spot 17; Table 7.5) showed an expression increase of 2.3-fold and returned as the tyrB gene product by BLASTp analysis (E = 0). TyrB is involved in the biosynthesis of aromatic amino acids and was also identified in section 5.4 as increased in AB210 *vs.* AB211.

Pyruvate dehydrogenase (Spot 18; Table 7.5) showed an expression increase of 2.2-fold and has been previously identified in section 7.4.2 as increased in both SM346 and NCTC 10211.

7.7 Chapter Summary

The aim of this investigation was to characterise the differentially expressed proteins upon upregulation of the *S. marcescens* efflux pump SdeXY. However, unlike in previous chapters, a pair of isolates was not used as the tigecycline-resistant clinical isolate SM346 did not have a tigecycline-susceptible counterpart. This was a major caveat in this investigation, as it was difficult to interpret the protein results given the isolates' dissimilarity. This led to differentially expressed proteins being identified that were unrelated to the resistance mechanism.

However, using the tigecycline-susceptible type strain NCTC 10211 and its -resistant derivative 10211-10, the results from the DIGE comparisons demonstrate a diverse range of proteins affected. As these isolates were closely related, it allowed the changes more likely associated with resistance to be highlighted. Efflux upregulation in 10211-10 may be associated with an increase in proteins that have the potential to increase virulence (PhoP) and iron acquisition (FepA). PhoP can also confer increased survival under low magnesium, low pH and the presence of polymyxin B (Barchiesi *et al.* 2012). PhoP mutants are also defective for survival in epithelial cells, which may be associated with control of these virulence phenotypes. Iron acquisition has shown to be a crucial process for *S. marcescens* infection in a *Caenorhabditis elegans* model (Kurz *et al.* 2003).

Expression increases in 10211-10 were observed for proteins involved in cell adhesion (DegP) and biofilm formation (GmhA) *vs.* SM346. DegP is an essential virulence factor for many pathogens, for instance it is required for enteropathogenic *E. coli* (EPEC) pathogenesis, as it chaperones and assembles the fimbrial adhesins which confer bacterial attachment (Humphries *et al.* 2011), DegP is also required for full virulence in *S. pyogenes* and reduces susceptibility to ROS (Jones *et al.* 2001). GmhA is required for biofilm formation in *Yersinia pestis* (Darby *et al.* 2005) and also for biosynthesis of the LPS inner core in *E. coli*.

Another protein with a potential role in LPS biosynthesis, GalU, was identified in section 7.5 in SM346 *vs.* 10211-10. Klein *et al.* showed that GalU mutants of *Y. pestis* attenuated survival in murine macrophages and caused the formation of truncated lipooligosaccharides, suggesting its importance in LPS formation (Klein *et al.* 2012). Nesper *et al* showed GalU is required for LPS

biosynthesis and biofilm formation in *Vibrio cholerae* (Nesper *et al.* 2001). The increase of these proteins in both of the tigecycline-resistant isolates SM346 and 10211-10 suggests that LPS biosynthesis may be associated with upregulated efflux in *S. marcescens*.

In previous comparisons, many tigecycline-resistant clinical isolates showed reductions in metabolic proteins compared with their susceptible counterparts. However, SM346 was shown to have higher expression of many metabolic proteins compared with NCTC 10211, possibly because as a clinical isolate, SM346 may have adapted its metabolism to cope with decreased nutrient availability (in the host environment). These differences were reflected in the comparison with 10211-10, where SM346 again showed relative increases in many metabolic proteins.

Stress defence proteins in 10211-10 demonstrate large increases in protein expression, while fewer proteins increased expression in the clinical isolate. This suggests that SM346 has alternative mechanisms for dealing with stress or that it has adapted to the protein expression changes caused by upregulation of the SdeXY efflux pump. For instance, GroEL showed large increases in both tigecycline-resistant isolates and could be utilised in stress defence in *S. marcescens*. As the clinical isolate SM346 demonstrated increased levels of metabolic proteins, possibly due to differences in its environment and nutrients, this may explain the concomitant increase in stress defence proteins.

Other changes in *S. marcescens* included the Min proteins, which may have potential to be a novel target for the treatment of MDR Gram-negative pathogens and needs further work to confirm whether it has an active role in efflux-mediated resistance in *S. marcescens*. The increased expression of CytR in SM346 confirms the increased biofilm-forming capabilities of 10211-10. CytR is known to repress biofilm formation in *Vibrio cholerae* (Haugo & Watnick 2002) and was increased in SM346 *vs.* 10211-10 *i.e.* a lower level of CytR in 10211-10 may actually provide a relative increase in biofilm formation. SM346 displays expression changes in proteins involved in membrane transport *e.g.* the Sec transport system, lipid transporter and an ABC carbohydrate transporter, involving rearrangement of the outer membrane proteome.

Many changes have been identified in 10211-10, some of which may be attributed to the upregulation of SdeXY, such as increased expression of proteins involved in biofilm formation, iron acquisition and LPS biosynthesis. These are changes that could potentially make this organism more virulent and could further complicate treatment. However, the changes identified in SM346 were more difficult to explain without an isogenic comparator and because of this, few changes could confidently be attributed to efflux upregulation due to the genetic dissimilarity between SM346 and the 10211 isolates. An example of these difficulties included the potential presence of protein isoforms *e.g.* pyruvate dehydrogenase, which showed increased expression in both SM346 and NCTC 10211 in the same comparison. Differences in the respective complements of stress response and metabolic proteins, confirms the anticipated difficulties of comparing genetically unrelated isolates. Due to these difficulties, the results were more difficult to interpret than those for *Enterobacter* and *Acinetobacter.*

This is the first work to investigate the proteomic changes associated with upregulated efflux in *S. marcescens* and a number of proteins from different functional groups have been identified. This chapter has highlighted the potential difficulties when using DIGE on unrelated organisms and ideally, in future only isogenic pairs of isolates or different conditions applied to the same isolate should be tested.

8. General Discussion

The overall aim of this study was to characterise the proteins involved in the selected antibiotic resistance mechanisms, using proteomics approaches such as 2DGE, quantitative labelling and mass spectrometry. While the modes of action of these drugs have been elucidated, there was also an underlying objective, to ascertain whether proteomics could be used in tandem with current molecular techniques to probe the broader implications on bacterial cell physiology.

Three key antimicrobial resistances of public health importance were investigated:

1) Plasmid-mediated multidrug-resistance in *E. coli*

2) Non-carbapenemase-mediated carbapenem resistance in *K. pneumoniae*

3) Efflux-mediated tigecycline resistance in *A. baumannii*, *E. cloacae* and *S. marcescens*.

This investigation identified a plethora of proteins with functions that relate to antibiotic resistance, virulence and many other functional classes involved in general bacterial cell physiology. Some of these could not have been defined without the use of modern MS-based proteomics *e.g.* the analysis of the OMPs of *K. pneumoniae* or the quantification of differential protein expression using DIGE. The differentially expressed proteins were assigned explanations for why they were differentially expressed, present or absent in an attempt to assess their role in, or their relevance to, the mechanism of resistance. The results obtained from each resistance-organism combination reveal many further avenues for investigation and are detailed later in this chapter.

There are many proteomic methods available and a selection was used in these investigations. Below is a short review of the techniques used and whether they may find future applications in clinical laboratories.

2DGE was used to separate the proteins from whole-cell extracts and identify the digested peptides, initially with MALDI-TOF MS. 2DGE is useful for obtaining protein identifications as the excised spots are likely to release sufficient peptides upon digestion for MS analysis and protein identification. Due to its large-scale coverage of the proteome, 2DGE is an effective technique for detecting differences in the spot profile of expressed proteins and is still in use today

(Marzoa *et al.* 2012). Because of its ease in visualising differences between profiles, it is often applied at the preliminary stages of comparative differential expression profiling of microorganisms. Additional advantages of DIGE include pooling the labelled samples and running them in one gel along with internal standardisation, which removes the problems associated with gel-to-gel variation which plague analysis of conventional 2DGE gels. Some of the disadvantages of DIGE are that although a lot of information can be gleaned from one experiment, there is no guarantee that the proteins of interest will be expressed (and detected) at a high enough level for downstream analysis. Also, many proteins which appear as 'unique' to one isolate may not be expressed, rather than demonstrating the loass of a protein, making interpretation of the expression profiles more difficult. For example, in the comparison of *A. baumannii* isolates AB210-6/AB211, a protein highlighted as 'unique' to one isolate was later identified as differentially expressed between both isolates. Although this could be a software error or a novel form of the same protein, such as a post-translationally modified isoform of the protein.

DIGE also requires closely-related isolates to compare with the resistant isolate, otherwise any potential changes are much harder to elucidate from the data. For instance, the differences in expression identified in *Serratia* isolate SM346 were difficult to explain without an isogenic comparator. Because of this, few changes could confidently be attributed to efflux upregulation due to the dissimilarity between SM346 and the 10211 isolates. Due to these differences, the *S. marcescens* DIGE results were more difficult to interpret then those for *Enterobacter* and *Acinetobacter*.

To attempt to characterise the proteins in these antibiotic resistant isolates in the first instance, a discovery proteomics approach was used which generates a vast amount of data and highlights changes that are not necessarily attributed to the condition being studied. Therefore, a significant amount of effort and time is dedicated to analysis in order to extract the relevant features from the data. For this reason it is necessary to apply targeted approaches to further confirm that the proteins of interest are truly associated with resistance. This would eliminate a lot of uncertainty and increase confidence in the results, making the techniques more amenable to a reference laboratory.

When 2DGE is combined with MALDI-TOF, in the absence of other higher resolution techniques, the major proteins visualised on a gel may be rapidly identified in minutes. At the commencement of this study, this was the only accessible technique. However, with the subsequent arrival of an LC-MS/MS system, it became possible to run SDS-PAGE gels, excise multiple bands and subject them to MS/MS analysis (designated GeLC-MS/MS) to extend the range of proteins identified. Here 2DGE was useful for identifying single proteins expressed from the transformants, at the early stages of this study. The technique has limitations in that it is very labour intensive, requires considerable technical skills and proteins that are not visualised in the gels may missed. Also, as 2DGE only identifies single proteins, the GeLC-MS/MS approach is more suitable and has the potential to profile the expressed proteins which are affected by the development of antimicrobial resistance.

Due to the low sensitivity of MALDI-TOF Also, as the approach only identifies single proteins, the GeLC-MS/MS approach is much more suitable and has the potential to profile the expressed resistance proteins. While more expensive and more technically demanding to operate, the nano LC-LTQ Orbitrap considerably higher resolution than MALDI-TOF. In general, the former provides large dynamic range, high mass accuracy and is able to process complex samples (Graham *et al.* 2011). LC-MS/MS proved a powerful technique in the GeLC analysis, returning hundreds of identifications from the gel profiles of whole-cell extracts. GeLC-MS/MS is also useful in detecting lower abundance proteins that 2DGE/DIGE may have missed.

The GeLC approach proved useful to map the exact changes in *K. pneumoniae* OMP composition between a clinical pair of isolates and also to probe differences between J53 and derivative transformants. The potential of this technique is reinforced by its application in profiling organisms for their expressed resistance profile *e.g.* CTX-M-15 was detected in both the *Klebsiella* isolates and CTX-M-3 was detected in both the *E. coli* transformants.

The Biolog System, which provides global coverage of the phenotype of an unknown microorganism, was utilised here to facilitate broader coverage of changes upon plasmid acquisition. Furthermore, the inclusion of antimicrobial agents against an appropriate control for each test substrate enables a vast number of antimicrobial agents to be tested simultaneously. The elucidation of phenotypes by this method provided considerable data and was useful in interpreting

and validating the proteomic data. However, the full run of 20 phenotype microarray plates was excessive, as there were few plates of relevance to the study of resistance mechanisms. The plates that were available contained many old or dated compounds *e.g.* some of the biocides used had very little information available, while for others the mechanisms of inhibition are poorly characterised. However, the method has some advantages for probing phenotypic differences between isolates/pairs. As well as for elucidating changes in organisms after gene knockout and could be recommended for studies of this nature, particularly in combination with metabolomic approaches. In the future, a more flexible system in which well characterised antimicrobial mechanisms could be selected and tested in parallel with transcriptomics and proteomics may prove useful as a novel approach to elucidating complex mechanisms of resistance in microorganisms.

These approaches generated a lot of data on the presence/absence of proteins and expressed protein profiles. However, quantitative proteomics was able to further probe the more subtle protein changes in both antibiotic-susceptible and -resistant organisms. The large amount of data generated in these chapters also raised many possibilities of further study on the resistances of these organisms. These are described in detail, after a summary of the results of this PhD in each of the organisms investigated.

Escherichia coli

It is known that the acquisition of a plasmid can affect the protein composition of a cell, such as alteration of porin levels (Russell, 1997). Two transformants were subjected to whole-cell proteome analysis to identify any changes in the expressed proteome. While more subtle changes in expression could not be detected, the presence or absence of proteins were detected and compared with J53, the host of the two resistance plasmids. The technique was able to identify proteins present only in J53 and proteins only present in J204 and J499, some of which were expressed by the plasmid and some were chromosomally located. The combined proteomics and phenomics approaches allowed the identification of proteins involved in membrane integrity (the Tol-Pal system), changes in which may be responsible for the altered phenotypes identified by PM analysis

e.g. increased biocide resistance and increased polymyxin susceptibility. The results from the GeLC-MS/MS experiments also opens the possibility of plasmid profiling by proteomics, to ascertain the percentage of proteins expressed from a plasmid; and subsequent resistance profiling, to identify as many resistant proteins expressed as possible.

Klebsiella pneumoniae

The use of SDS-PAGE to separate and visualise the OMP profiles of *K. pneumoniae* isolates 1A and 1B, successfully confirmed the predicted resistance mechanism of reduced porin expression (of Ompk35 and OmpK36) combined with ESBL-production in 1B (Webster *et al.* 2010). Further analysis by LC-MS/MS yielded a unique perspective on OMP changes in carbapenem-resistant *K. pneumoniae*. In particular, changes were observed which suggested the resistant isolate may fare less well in an infection model, as it lacked proteins required for colonisation of a host (*e.g.* SuhA, FimA, -C, -D, -F and TraT). 1B also lacked the colicin-type Klebicin B, which may allow it to be outcompeted by other isolates. Interestingly, isolate 1B expressed OmpK26 which identified as KdgM, a porin potentially used by the Tog oligosaccharide transport system. OmpK26 has previously been identified as expressed in carbapenem-resistant *Enterobacter*iaceae with repressed OmpK35/36 porins (García-Sureda *et al.* 2011) and was confirmed in this study in carbapenem-resistant isolate 1B. There were also some additional antibiotic resistance proteins expressed only in 1B, including; EmrA, periplasmic component part of a MDR efflux pump and APH(3''), involved in streptomycin resistance. These results pose many questions regarding the effects of these protein changes and require additional studies.

As with many of the isolates described in this thesis, (with the exceptional of the *A. baumannii* clinical pair) it would have been very helpful to have had their genomes sequenced, to confirm whether any of the observed presence/absence of proteins or increase/decrease in expression are due to changes in the genome or whether they are purely just protein expression differences.

Acinetobacter baumannii

Based upon the initial analyses of the clinical pair, AB210 and AB211, there were some proteins which were identified in one of the isolates and not the other. These proteins included AAC(6')-Ib, identified only in AB210 and was confirmed with genome sequencing data (Hornsey *et al.* 2011) and the reduction in AB211 aminoglycoside MICs (see methods section 2.2; Table 2.2). There were other proteins identified in just one isolate, however, they could not be confirmed by genome sequencing. This highlights a major caveat in 2DGE/DIGE as from the presence/absence of one spot, there is no way to tell if there are isoforms of the protein in both isolates or the protein was not abundant enough in one isolate. It also highlights the value of having a genome sequence to confirm the changes reported by proteomics.

The DIGE approach highlighted many differences between the pair of isolates (Tables 5.2 and 5.3; section 5.4) including proteins which could potentially give AB211 an advantage over AB210 in an infection. These included proteins such as ferrichrome iron receptor protein and PldA, involved in cell envelope biogenesis and colonisation (Istivan & Coloe, 2006). NDK was also found to be reduced in AB211 compared with AB210, which can cause elevated rates of mutation when suppressed (Miller *et al.* 2002). In AB211, this protein was found to have a mutation by whole genome sequencing which could have increased this isolate's mutation rate (Hornsey *et al.* 2011). Similar results were found in the other comparisons with mutant derivatives of the pair, for instance proteins increased in AB210-6 (a tigecycline resistant mutant) included a polysaccharide biosynthesis protein and ferrichrome iron receptor (the same as identified in AB211). These results also highlighted how the two different acquisitions of resistance in these two resistant mutants had different effects on their protein profiles.

However, while many results were obtained from the DIGE technique, it wasn't always successful. Although AB210 and AB210-6 were compared, not enough protein identifications were returned to make reliable inferences about the significance of proteins displaying differential expression. Even with high quality gels with good separation applied to pairs of isolates, DIGE does not guarantee detection of all differentially expressed proteins.

Enterobacter cloacae

This study aimed to characterise the changes in the protein profile of the *E. cloacae* isolates upon upregulation of AcrAB-TolC efflux pump conferring resistance to tigecycline. Some of the proteins identified as differentially expressed between the isolates displayed positive associations with AcrAB activity. The expression of these proteins increased when *acrAB* was upregulated, and some displayed negative associations; expression of these proteins were reduced when *acrAB* was upregulated. Those that demonstrated a positive association were OmpD, a porin implicated in stress resistance and SdhA or succinate dehydrogenase subunit A (section 6.3.1). While the latter was likely involved in supplying energy to the efflux pump, the contribution of OmpD is less well understood, although other studies suggest it is involved in resistance to heat (Ruan *et al.* 2011) and antibiotics (Szabó *et al.* 2006; Pilonieta *et al.* 2009)

However, there were also some proteins which, upon modulation of expression, may play a role in the virulence of these isolates such as OmpA and LuxS. These proteins did not show any association of expression with AcrAB but their expression was changed upon *acrB* knockout, regulation of AcrB can affect the virulence of this organism. Alternatively, these proteins may have differences in expression due to a key membrane component being lost and needs to be investigated further.

Serratia marcescens

Few results from the comparisons with SM346 could be interpreted or discussed further as there were no genetically similar isolates to compare against. The 10211 type strain and its derivatives were all highly dissimilar to SM346, therefore any observed differences in protein expression may not have been caused by the upregulation of SdeXY, but may arise from pre-existing genetic differences between isolates. However, the comparison of NCTC 10211 and its tigecycline-resistant laboratory derivative 10211-10 did reveal changes in protein expression. Changes in proteins such as PhoP, GmhA and FepA were identified, which code for virulence phenotypes, LPS biosynthesis and iron acquisition respectively (Barchiesi *et al.* 2012; Kneidinger *et al.* 2002; Newton *et al.* 2010). In future studies, lone resistant organisms should not be analysed with

proteomics, as an isogenic comparator is required to reduce the background 'noise' of unrelated protein changes.

Some proteins were consistently identified in the DIGE experiments as differentially expressed across all the species tested. The Min proteins may be important for efflux upregulation as changes in the MinCDE proteins were observed in almost every comparison of isolates from chapters 5, 6 and 7. Min proteins show differential regulation in almost every comparison in; *Acinetobacter, Enterobacter* and *Serratia* DIGE chapters, with MinC and MinD most commonly identified. MinC is the inhibitor of septum formation and MinD its regulator. This suggests that the upregulation of major efflux pump genes has an effect on the cellular division processes, or may be regulated by the same system, that may be conserved across bacterial genera. Although, given its essential function in the regulation of cell division, Min may not have any role in the upregulation of efflux. In either case, the role of these proteins is worth further investigation in the context of efflux upregulation.

8.1 Future work

There is a vast potential for further work arising from this investigation. Due to the number of different organisms, the breadth of antibiotic resistance mechanisms and the range of techniques used, which even individually, generated large amounts of data.

To validate any conclusions drawn from the proteins highlighted by the GeLC experiment from the J53/transformant comparison, further work needs to be done on these proteins to ascertain their precise role in modulation of the host cell proteome upon plasmid acquisition *e.g.* TolA, Pal, YbgF. For instance, mutants need to be generated lacking these proteins and the experiment would be repeated to see if susceptibilities have changed. Further work also needs to be done on transformant susceptibilities to biocides and antiseptics, because although there are no specific resistance genes on the plasmids for tolerance to antiseptics, the PM results show the transformants have an advantage in the presence of certain agents. It would also be pertinent to elucidate whether different

plasmids can confer similar phenotypes and protein profiles and requires testing of a much larger panel of transformants carrying a range of plasmids, all in J53.

One control which was never implemented in the *E. coli* experiments was to compare J53 (with no plasmid) against electroporated J53 (with no plasmid), to see if the stress of electroporation had any residual effect on OMP composition of transformants. Quantification experiments on these isolates to ascertain the subtler changes caused by plasmid acquisition would also have been valuable, but due to time constraints, they were never undertaken.

From the investigation into the *K. pneumoniae* pair of isolates, the identification of OmpK26 in 1B poses as a potential marker for OmpK35/36 porin loss. To further investigate this requires screening for the presence of OmpK26 in other non-carbapenemase-mediated carbapenem resistance isolates. Related to this protein is the expression of the Tog multi-component membrane complex, which is as yet unknown to be involved in antibiotic resistance and also requires further study. Many proteins functioning as virulence factors were not identified in 1B such as FhuA and fimbrial proteins FimA, B, C, F. It would be worth testing whether 1B is outcompeted by 1A in an infection model, to see if this particular mechanism is an advantage or disadvantage *in vivo*. With the lack of these factors in additiono to the Klebicin B protein, 1B may be outcompeted by other isolates and could be tested by co-infection in an *in vivo* model.

As described previously, this pair would ideally be sequenced allowing higher-confidence proteo-genomic comparisons. This should allow the confirmation of the origin of the protein changes between the pair *e.g.* whether they are protein expression differences or genetic changes. Also, analysis of the whole-cell extracts of transformants and *K. pneumoniae* isolates should be repeated on a newer, more sensitive LC-MS/MS. It is thought that a more sensitive analytical instrument would be able to detect the peptides required (those containing the amino acid change or changes) to comprehensively differentiate resistance enzymes. For example, to discern CTX-M enzymes from one another, which would provide further evidence for the application of LC-MS/MS in the resistance profiling of isolates.

To complete the comprehensive analysis of the *A. baumannii* isolates, AB210-6 and AB211Δ*adeB* should be submitted for genome sequencing, allowing genetic confirmation of protein changes and consistency (in terms of genetic conformation) across the four isolates used.

Specific further tests include *in vivo* infection models to test if AB211 could outcompete AB210, with its increase in virulence-associated factors expressed. More specifically, to see if these expression changes actually translated into a phenotypic difference, the identified virulence factors should be tested with assays for iron acquisition (investigate growth in broth with an iron chelator); cell attachment assays (incubate labelled bacteria with eukaryotic cells, compare attachment levels of clinical pair of isolates); and biofilm formation (measure levels of biofilm growth and integrity by challenging the biofilm with antibiotics and measuring cell death). A similar approach should be taken with AB210-6, whereby these assays are repeated to confirm whether AB210 has a similar phenotype to AB211, if so, then it is more likely that efflux upregulation is responsible for the observed changes.

It was observed that many of the changes between the AB210 and AB211 were in proteins residing on the OM, the next step in their proteome characterisation should be OMP analysis. It was successfully utilised when analysing the OMPs of *K. pneumoniae* isolates and identified a large proportion of the proteins as virulence-associated. Given that many differences between the *A. baumannii* pair were also virulence associated, OM analysis would provide additional information on the differences between the isolates.

As with other isolates in this research project, the *E. cloacae* pair and mutants should have their genomes sequenced to confirm changes in protein expression. It would also be worth comparing knockout mutants of OmpD to determine its function in this pair, to investigate whether a TGC-RΔ*ompD* mutant would have the same level of resistance as TGC-S, which could suggest if OmpD is required for efflux-mediated resistance or not. Also, it was hypothesised that modulation of some proteins identified (OmpA and LuxS) could alter the virulence properties of the isolates. This also requires investigation, to determine whether the upregulation of AcrAB could give an *in vivo* advantage. As well as determining if the resistant organism could outcompete the susceptible organism, or whether the isolate could be outcompeted.

The isolates being compared to the tigecycline-resistant clinical isolate SM346 were not isogenic comparators, so there were few results to interpret and therefore fewer findings to follow up on. However, comparison with NCTC 10211 and its derivatives revealed changes in protein expression similar to those seen in previous *Acinetobacter* comparisons. These include iron acquisition proteins and biofilm-forming proteins, the role of which should be investigated in a similar manner to the proteins in *Acinetobacter*.

8.2 Conclusions

This research project has contributed to the ever-expanding knowledge base of microbial proteomics, in this case: towards three resistances of clinical relevance, in five pathogenic organisms with public health importance. It has been demonstrated that some techniques are in-line with the work of resistance reference laboratories. While many advantages of proteomics have been presented, it is clear that proteomics should be used in combination with other approaches. As the use of genomics, proteomics and metabolomics in tandem will allow global profiling of microorganisms. This allows a more thorough characterisation of results due to additional confirmations of protein expression differences through mutations in the genome and altered metabolome profile.

As the caveats of proteomics are addressed, such as a greater representation of low-abundance proteins and higher coverage of the expressed proteome, the techniques are becoming more established. As the sensitivity and resolution of MS instruments improves, the applications of proteomics technologies will continue to rise.

From all the results obtained, the key findings revealed by this research project are summarised below:

- Plasmid acquisition has the potential to increase tolerance to certain biocides but may also increase susceptibility to polymyxin antibiotics and other agents (2-phenylphenol), these changes are potentially connected with changes found in the OMPs from these isolates.

- Non-carbapenemase-mediated Carbapenem resistance in *Klebsiella pneumoniae* has the potential to reduce virulence factor production but induce additional resistance factors. Expression of OmpK26 in carbapenem-resistant 1B confirmed by proteomics techniques.

- The GeLC-MS/MS technique identified CTX-M-3 in the plasmid-harbouring J53 isolates and also CTX-M-15 in the *K. pneumoniae* isolates. While J499 actually produced CTX-M-15 and the *K. pneumoniae* isolates specific CTX-M was not known (the PCR only tested for a group 1 CTX-M enzyme), with more powerful MS/MS instrument, more peptides could be detected increasing the chances of precise ESBL identification.

- In *A. baumannii* changes between the pair of isolates were confirmed with the analysis of mutant derivatives AB210-6 and AB211Δ*adeB*. Proteomics described many changes in the isolate AB211, potentially conferring greater ability to scavenge iron, form biofilms and cause infection in a host.

- In *E. cloacae*, OmpD and SdhA were identified as displaying a positive expression association with the efflux protein AcrB. These proteins have not previously been identified as displaying increased expression in tigecycline-resistant *E. cloacae* and require further testing as potential markers for this mechanism.

- The DIGE results also identified proteins such as the Min cell division proteins, which displayed differential expression across comparisons of *Acinetobacter*, *Enterobacter* and *Serratia*. There were also iron acquisition and biofilm-forming proteins increased in resistant *vs.* susceptible isolates in both *A. baumannii* and *S. marcescens*.

References

Abbott, D. W. & Boraston, A. B. (2008) Structural biology of pectin degradation by Enterobacteriaceae. *Microbiology and Molecular Biology Reviews* 72, 301-316.

Adams, M. A. & Jia, Z. (2006) Modulator of drug activity B from *Escherichia coli*: crystal structure of a prokaryotic homologue of DT-diaphorase. *Journal of Molecular Biology* 359, 455-65.

Aebersold, R. & Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* 422, 198-207.

Agafonov, D. E. & Spirin, A. S. (2004) The ribosome-associated inhibitor A reduces translation errors. *Biochemical and Biophysical Research Communications* 320, 354-8.

Al-Shahib, A., Misra, R., Ahmod, N., Fang, M., Shah, H. & Gharbia, S. (2010) Coherent pipeline for biomarker discovery using mass spectrometry and bioinformatics. *BMC Bioinformatics* 11, 437.

Albrethsen, J. (2007) Reproducibility in protein profiling by MALDI-TOF mass spectrometry. *Clinical Chemistry* 53, 852-8.

Alekshun, M. N. & Levy, S. B. (2007) Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128, 1037-50.

Allali, N., Afif, H., Couturier, M. & Melderen, L. V. (2002) The highly conserved TldD and TldE Proteins of *Escherichia coli* are involved in Microcin B17 processing and in CcdA degradation. *Journal of Bacteriology* 184, 3224-3231.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J. & Handelsman, J. (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology* 8, 251-9.

Alteri, C. J. & Mobley, H. L. T. (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. *Infection and Immunity* 75, 2679-88.

Alteri, C. J., Smith, S. N. & Mobley, H. L. T. (2009) Fitness of *Escherichia coli* during urinary tract infection requires gluconeogenesis and the TCA cycle. *PLoS pathogens* 5, e1000448.

Andrews, J. & Howe, R. A. (2011) BSAC standardized disc susceptibility testing method (version 10). *Journal of Antimicrobial Chemotherapy* 66, 2726-57.

Armengod, M., Moukadiri, I., Prado, S., Ruiz-partida, R., Benítez-páez, A., Villarroya, M., Lomas, R., Garzon, M. J., Martinez-Zamora, A., Meseguer, S. & Navarro-Gonzalez, C. (2012) Enzymology of tRNA modification in the bacterial MnmEG pathway. *Biochimie* 94, 1510-1520.

Aziz, R. K., Breitbart, M. & Edwards, R. A. (2010) Transposases are the most abundant, most ubiquitous genes in nature. *Nucleic Acids Research* 38, 4207-17.

Bailey, A. M., Paulsen, I. T. & Piddock, L. J. V. (2008) RamA confers multidrug resistance in Salmonella enterica via increased expression of acrB, which is inhibited by chlorpromazine. *Antimicrobial Agents and Chemotherapy* 52, 3604-11.

Baltes, N., Hennig-Pauka, I., Jacobsen, I., Gruber, A. D. & Gerlach, G. F. (2003) Identification of dimethyl sulfoxide reductase in Actinobacillus pleuropneumoniae and its role in infection. *Infection and Immunity* 71, 6784-6792.

Bandyopadhyay, K., Parua, P. K., Datta, A. B. & Parrack, P. (2010) *Escherichia coli* HflK and HflC can individually inhibit the HflB (FtsH)-mediated proteolysis of kCII in vitro. *Archives of Biochemistry and Biophysics* 501, 239-243.

Barchiesi, J., Castelli, M. E., Di, G., Colombo, M. I. & García, E. (2012) The PhoP/PhoQ System and its role in *Serratia marcescens* pathogenesis. *Journal of Bacteriology* 194,2949-6

Bartoloni, A., Pallecchi, L., Rodríguez, H., Fernandez, C., Mantella, A., Bartalesi, F., Strohmeyer, M., et al. (2009) Antibiotic resistance in a very remote Amazonas community. *International Journal of Antimicrobial Agents 33*, 125-129.

Bateman, S. L. & Seed, P. C. (2012) Epigenetic regulation of the nitrosative stress response and intracellular macrophage survival by extraintestinal pathogenic *Escherichia coli. Molecular Microbiology* 83, 908-925.

Bean, D. C., Krahe, D. & Wareham, D. W. (2008) Antimicrobial resistance in community and nosocomial *Escherichia coli* urinary tract isolates, London 2005 – 2006. *Annals of Clinical Microbiology and Antimicrobials* 7, 3-6.

Becker, C. H. & Bern, M. (2011). Recent developments in quantitative proteomics. *Mutation Research* 722, 171-182.

Begic, S. & Worobec, E. A. (2008) Characterization of the *Serratia marcescens* SdeCDE multidrug efflux pump studied via gene knockout mutagenesis. *Pharmacia* 416, 411-416.

Beiras-Fernandez, A., Vogt, F., Sodian, R. & Weis, F. (2010) Daptomycin: a novel lipopeptide antibiotic against Gram-positive pathogens. *Infection and Drug Resistance* 3, 95-101.

Beloin, C., Valle, J., Latour-lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J. A. J., Molin, S., Prensier, G., Arbeille, B. & Ghigo, J. M. (2004) Global impact of mature biofilm lifestyle on Escherichia coli K-12 gene expression. *Molecular Microbiology 51*, 659-674.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology* 153 Suppl, S347-57.

Benoit, S., Abaibou, H. & Mandrand-Berthelot, M. A.(1998) Topological analysis of the aerobic membrane-bound formate dehydrogenase of *Escherichia coli. Journal of Bacteriology* 180, 6625-6634.

Blair, J. M. A., La Ragione, R. M., Woodward, M. J. & Piddock, L. J. V. (2009) Periplasmic adaptor protein AcrA has a distinct role in the antibiotic resistance and virulence of *Salmonella enterica* serovar Typhimurium. *Journal of Antimicrobial Chemotherapy* 64, 965-72.

Bochner, B. R., Gadzinski, P. & Panomitros, E. (2001) Phenotype Microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Research* 11, 1246-1255.

Bompard-Gilles, C., Remaut, H., Villeret, V., Prange, T., Fanuel, L., Delmarcelle, M., Joris, B., Frere, J. & Van Beeumen, J. (2000) Crystal structure of a D-aminopeptidase from *Ochrobactrum anthropi*, a new member of the 'penicillin-recognizing enzyme' family. *Structure* 8, 971-980.

Bonnet, R. (2004) Growing group of Extended-Spectrum β-Lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy* 48, 1-14.

Bonsor, D. A., Hecht, O., Sharma, A., Krachler, M., Housden, N. G., Lilly, K. J., James, R., Moore, G. R. & Kleanthous, C. (2009) Allosteric β-propeller signalling in TolB and its manipulation by translocating colicins. *The EMBO Journal* 28, 2846-2857.

Brisse, S., Fevre, C., Passet, V., Issenhuth-jeanjean, S., Diancourt, L. & Grimont, P. (2009) Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS one* 4, e4982.

Brussow, H., Canchaya, C. & Hardt, W. (2004) Phages and the Evolution of Bacterial Pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews* 68, 560-602.

Buckel, W. & Golding, B. T. (2006) Radical enzymes in anaerobes. *Annual Review of Microbiology* 60, 27-49.

Burckhardt, I. & Zimmermann, S. (2011) Using matrix-assisted laser desorption ionization – time of flight mass spectrometry to detect carbapenem resistance. *Journal of Clinical Microbiology* 49, 3321-3324.

Bush, K. & Jacoby, G. A. (2010) Updated functional classification of β-lactamases. *Antimicrobial Agents and Chemotherapy* 54, 969-76.

Cabral, M. P., Soares, N. C., Parreira, R., Rumbo, C., Poza, M., Valle, J. & Calamia, V. (2011). Proteomic and functional analyses reveal a unique lifestyle for *Acinetobacter baumannii* biofilms and a key role for histidine metabolism. *Journal of Proteome Research* 10, 3399-3417.

Caldas, T. D., Yaagoubi, A. E. & Richarme, G. (1998) Chaperone properties of bacterial elongation factor EF-Tu. *Biochemistry* 273, 11478 -11482.

Calhoun, L. N. & Kwon, Y. M. (2011) Structure, function and regulation of the DNA-binding protein Dps and its role in acid and oxidative stress resistance in *Escherichia coli*: a review. *Journal of Applied Microbiology* 110, 375-86.

Camberg, J. L., Hoskins, J. R. & Wickner, S. (2011) The Interplay of ClpXP with the cell division machinery in *Escherichia coli*. *Journal of Bacteriology* 193, 1911-1918.

Cantón, R. & Coque, T. M. (2006) The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology* 9, 466-75.

Cantón, R., González-alba, J. M. & Galán, J. C. (2012) CTX-M enzymes: origin and diffusion. *Frontiers in Microbiology* 3: 110.

Carattoli, A. (2009) Resistance plasmid families in *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy* 53, 2227-2238.

Cardoso, K., Gandra, R. F., Wisniewski, E. S., Osaku, C. A., Kadowaki, M. K., Felipach-neto, V., Haus, L. F. & Simao, R. C. (2010). DnaK and GroEL are induced in response to antibiotic and heat shock in Acinetobacter baumannii Printed in Great Britain. *Journal of Medical Microbiology* 59, 1061-1068.

Carlone, G. M., Thomas, M. L., Rumschlag, H. S. & Sottnek, F. O. (1986) Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *Journal of Clinical Microbiology* 24, 330-332.

Carpousis, A. J. (2007) The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. *Annual Review of Microbiology* 61, 71-87.

Charani, E., Cooke, J. & Holmes, A. (2010) Antibiotic stewardship programmes - what's missing? *Journal of Antimicrobial Chemotherapy* 65, 2275-2277.

Chaudhuri, T. K., Verma, V. K. & Maheshwari, A. (2009) GroEL assisted folding of large polypeptide substrates in *Escherichia coli*: Present scenario and assignments for the future. *Progress in Biophysics and Molecular Biology* 99, 42-50.

Chen, J., Kuroda, T., Huda, N., Mizushima, T. & Tsuchiya, T. (2003) An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*. *Journal of Antimicrobial Chemotherapy* 52, 176-179.

Chng, S.-S., Ruiz, N., Chimalakonda, G., Silhavy, T. J. & Kahne, D. (2010) Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. *Proceedings of the National Academy of Sciences of the United States of America* 107, 5363-8.

Cho, Y.-seok, Jooyoung, J. & Oh, K.-heon. (2011) Synergistic anti-bacterial and proteomic effects of epigallocatechin gallate on clinical isolates of imipenem-resistant *Klebsiella pneumoniae*. *Phytomedicine* 18, 941-946.

Choi, C. H., Lee, J. S., Lee, Y. C., Park, T. I. & Lee, J. C. (2008) *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC Microbiology* 11, 1-11.

Ciesielski, S. J., Schilke, B. A., Osipiuk, J., Bigelow, L., Mulligan, R., Majewska, J., Joachimiak, A., Marszalek, J., Craig, E. A. & Dutkiewicz, R. (2012) Interaction of J-Protein co-chaperone Jac1 with Fe-S scaffold Isu is indispensable in vivo and conserved in evolution. *Journal of Molecular Biology* 417, 1-12.

Cohen-or, I., Shenhar, Y., Biran, D. & Ron, E. Z. (2010) CspC regulates rpoS transcript levels and complements hfq deletions. *Research in Microbiology* 161, 694-700.

Coldham, N. G., Randall, L. P., Piddock, L. J. V. & Woodward, M. J. (2006) Effect of fluoroquinolone exposure on the proteome of *Salmonella enterica* serovar Typhimurium. *Journal of Antimicrobial Chemotherapy* 58, 1145-53.

Cormican, M., Morris, D., Corbett-feeeney, G. & Flynn, J. (1998) Extended spectrum β-lactamase production and fluorquinolone resistance in pathogens associated with community acquired urinary tract infection. *Diagnostic Microbiology and Infectious Disease* 32, 317-319.

Costas, M., Holmes, B. & Sloss, L. L. (1990) Comparison of SDS-PAGE protein patterns with other typing methods for investigating the epidemiology of *Klebsiella aerogenes*. *Epidemiology and Infection* 104, 455-465.

Couce, A., Briales, A., Rodríguez-rojas, A., Costas, C., Pascual, Á. & Blazquez, J. (2012) Genomewide overexpression screen for fosfomycin resistance in *Escherichia coli*: MurA confers clinical resistance at low fitness cost. *Antimicrobial Agents and Chemotherapy* 56, 2767-69.

Croucher, N. J. & Thomson, N. R. (2010) Studying bacterial transcriptomes using RNA-seq. *Current Opinion in Microbiology* 13, 619-624.

Croxatto, A., Prod'hom, G. & Greub, G. (2011) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews* 36, 380-407.

Dalben, M., Varkulja, G., Basso, M., Krebs, V. L. J., Gibelli, M. A., van der Heijden, I., Rossi, F., Duboc, G., Levin, A. S. & Costa S. F. (2008) Investigation of an outbreak of *Enterobacter cloacae* in a neonatal unit and review of the literature. *Journal of Hospital Infection* 70, 7-14.

Damron, F. H., Davis Jr, M. R., Withers, T. R., Ernst, R. K., Goldberg, J. B., Yu, G. & Yu, H. D. (2012) Vanadate and triclosan synergistically induce alginate production by *Pseudomonas aeruginosa* strain PAO1. *Molecular Microbiology* 81, 554-570.

Dangi, B., Gronenborn, A. M., Rosner, J. L. & Martin, R. G. (2004) Versatility of the carboxy-terminal domain of the alpha subunit of RNA polymerase in transcriptional activation: use of the DNA contact site as a protein contact site for MarA. *Molecular microbiology* 54, 45-59.

Darby, C., Ananth, S. L., Tan, L. & Hinnebusch, B. J. (2005) Identification of *gmhA*, a *Yersinia pestis* gene required for flea blockage, by using a *Caenorhabditis elegans* biofilm system. *Infection and Immunity* 73, 7236-7242.

Datta, N. & Kontomichalou, P. (1965) Penicillinase synthesis controlled by infectious R factors in *Enterobacteriaceae*. *Nature* 208, 239-41.

David, M. D., Weller, T. M. A., Lambert, P. & Fraise, A. P. (2006) An outbreak of *Serratia marcescens* on the neonatal unit: a tale of two clones. *Journal of Hospital Infection* 63,27-33.

Davies, J. & Wright, G. D. (1997) Bacterial resistance to aminoglycoside antibiotics. *Trends in Microbiology* 5, 234-240.

Dhanji, H., Patel, R., Wall, R., Doumith, M., Patel, B., Hope, R., Livermore, D. M. & Woodford, N. W. (2011) Variation in the genetic environments of bla CTX-M-15 in Escherichia coli from the faeces of travellers returning to the United Kingdom. *Journal of Antimicrobial Chemotherapy* 66, 1005-1012.

Dibner, J. J. & Richards, J. D. (2005) Antibiotic growth promoters in Agriculture: history and mode of action. *Poultry Science* 84, 634-643.

Dijkshoorn, L., Nemec, A. & Seifert, H. (2007) An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nature Reviews Microbiology* 5, 939-51.

dos Santos, K. V., Diniz, C. G., Veloso, L. D. C., de Andrade, H. M., Giusta, M. D. S., Pires, S. D. F., Santos, A. V., Apolonio, A. C. M., de Carvalho, M. A. R. & Farias, L. deM. (2010) Proteomic analysis of *Escherichia coli* with experimentally induced resistance to piperacillin/tazobactam. *Research in Microbiology* 161, 268-75.

Doumith, M., Ellington, M. J., Livermore, D. M. & Woodford, N. (2009) Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. *Journal of Antimicrobial Chemotherapy* 63, 659-67.

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N. & Wright, G. D. (2011) Antibiotic resistance is ancient. *Nature* 31, 457-461.

D'Costa, V. M., Mukhtar, T. A., Patel, T., Koteva, K., Waglechner, N., Hughes, D. W., Wright, G. D. & DePascale, G. (2012) Inactivation of the lipopeptide antibiotic daptomycin by hydrolytic mechanisms. *Antimicrobial Agents and Chemotherapy* 56, 757-764.

Edelmann, M. J. (2011) Strong cation exchange chromatography in analysis of posttranslational modifications: innovations and perspectives. *Journal of Biomedicine and Biotechnology* 2011.

Elsemore, D. A. & Ornston, L. N. (1995) Unusual ancestry of dehydratases associated with quinate catabolism in Acinetobacter calcoaceticus. *Journal of Bacteriology* 177, 5971-8.

Encheva, V., Gharbia, S. E., Wait, R., Begum, S. & Shah, H. N. (2006) Comparison of extraction procedures for proteome analysis of *Streptococcus pneumoniae* and a basic reference map. *Proteomics* 6, 3306-17.

Falagas, M. E. & Bliziotis, I. A. (2007) Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? *International Journal of Antimicrobial Agents* 29, 630-636.

Fancello, L., Desnues, C., Raoult, D. & Rolain, J. M. (2011) Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. *Journal of Antimicrobial Chemotherapy* 66, 2448-2454.

Fernandez-Alba, A. R., Piedra, L., Mezcua, M. & Hernando, M. D. (2002) Toxicity of single and mixed contaminants in seawater measured with acute toxicity bioassays. *Scientific World Journal* 25, 1115-1120.

Fernández-Reyes, M., Rodríguez-Falcón, M., Chiva, C., Pachón, J., Andreu, D. & Rivas, L. (2009) The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics* 9, 1632-45.

Fleming, A. (1945) Sir Alexander Fleming - Nobel Lecture: Penicillin. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-lecture.pdf. Last accessed on 19/09/2012

Fleming, A. (1947) Chemotherapy. Yesterday, Today and Tomorrow. The Linacre lecture. *British Journal of Surgery* 34, 329.

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. & Dantas, G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337, 1107-1111.

Frankel, M. B. & Schneewind, O. (2012) Determinants of murein hydrolase targeting to cross-wall of *Staphylococcus aureus* peptidoglycan. *Plasmid* 287, 10460-71.

Frese, C. K., Altelaar, A. F. M., Hennrich, M. L., Nolting, D., Zeller, M., Griep-Raming, J., Heck, A. J. R. & Mohammed, S. (2011) Improved peptide identification by targeted fragmentation using CID , HCD and ETD on an LTQ-Orbitrap Velos. *Journal of Proteome Research* 10, 2377-2388.

Friedman, L., Alder, J. D. & Silverman, J. A. (2006) Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 50, 2137-45.

Fritsch, P. S., Urbanowski, M. L. & Stauffer, G. V. (2000) Role of the RNA polymerase α-subunits in MetR-dependent activation of metE and metH: important residues in the C-terminal domain and orientation requirements within RNA Polymerase. *Journal of Bacteriology* 182, 5539-5550.

Fritsche, T. R., Strabala, P. A., Sader, H. S., Dowzicky, M. J. & Jones, R. N. (2005) Activity of tigecycline tested against a global collection of *Enterobacteriaceae*, including tetracycline-resistant isolates. *Diagnostic Microbiology and Infectious Disease* 52, 209-13.

Frost, L. S., Leplae, R., Summers, A. O., Toussaint, A. & Edmonton, A. (2005) Mobile genetic elements: the agents of open source evolution. *Nature Reviews in Microbiology* 3, 722-733.

Fuda, C. C. S., Fisher, J. F. & Mobashery, S. (2005) β-Lactam resistance in *Staphylococcus aureus*. *Cellular and Molecular Life Sciences* 62, 2617-2633.

García-Sureda, L., Juan, C., Doménech-Sánchez, A. & Albertí, S. (2011) Role of *Klebsiella pneumoniae* LamB porin in antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* 55, 1803-5.

Gaupp, R., Schlag, S., Liebeke, M., Lalk, M. & Go, F. (2010) Advantage of upregulation of succinate dehydrogenase in *Staphylococcus aureus* biofilms. *Journal of Bacteriology* 192, 2385-2394.

Gilbert, D. N., Guidos, R. J., Boucher, H. W., Talbot, G. H., Spellberg, B., Edwards, J. E. J., Scheld, W. M., Bradley, J. S. & Bartlett, J. G. (2010) The 10 x '20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clinical Infectious Diseases* 50, 1081-83.

Gilbert, P. & Mcbain, A. J. (2003) Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clinical Microbiology Reviews* 16, 189-208.

Ginalski, K., Kinch, L., Rychlewski, L. & Grishin, N. V. (2004) BOF: a novel family of bacterial OB-fold proteins. *FEBS Letters* 567, 297-301.

Gomez, M. J. & Neyfakh, A. A. (2006) Genes involved in intrinsic antibiotic resistance of *Acinetobacter baylyi*. *Antimicrobial Agents and Chemotherapy* 50, 3562-7.

Gossert, A. D., Bettendorff, P., Puorger, C., Vetsch, M., Herrmann, T., Glockshuber, R. & Wüthrich, K. (2008) NMR structure of the *Escherichia coli* type 1 pilus subunit FimF and its interactions with other pilus subunits. *Journal of Molecular Biology* 375, 752-763.

Graham, C., Mcmullan, G. & Graham, R. L. J. (2011) Proteomics in the microbial sciences. *Bioengineered Bugs* 2, 17-30.

Graham, R. L. J., Sharma, M. K. & Ternan, N. G. (2007) A semi-quantitative GeLC-MS analysis of temporal proteome expression in the emerging nosocomial pathogen *Ochrobactrum anthropi*. *Genome Biology* 8, R110.

Graves, P. R. & Haystead, T. A. J. (2002) Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Reviews* 66, 39-63.

Gulmezian, M., Hyman, K. R., Marbois, B. N., Clarke, C. F. & Javor, T. (2008) The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Archives of Biochemistry and Biophysics* 467, 144-153.

Hamoen, L. W. (2011) Cell division blockage: but this time by a surprisingly conserved protein. *Molecular Microbiology* 81, 1-3.

Han, K. Y., Song, J. A., Ahn, K. Y., Park, J. S., Seo, H. S. & Lee, J. (2007) Enhanced solubility of heterologous proteins by fusion expression using stress-induced *Escherichia coli* protein, Tsf. *FEMS Microbiology Letters* 274, 132-138.

Hanlon, G. W. (2005) The emergence of multidrug resistant *Acinetobacter* species: a major concern in the hospital setting. *Letters in Applied Microbiology* 45, 375-8.

Hashimoto, C., Sakaguchi, K., Taniguchi, Y., Honda, H., Oshima, T., Ogasawara, N. & Kato, J. I. (2011) Effects on transcription of mutations in ygjD , yeaZ , and yjeE genes, which are involved in a universal tRNA modification in *Escherichia coli*. *Journal of Bacteriology* 193, 6075-6079.

Haugo, A. & Watnick, P. (2002) *Vibrio cholerae* CytR is a repressor of biofilm development. *Molecular Microbiology* 45, 471-483.

Hertle, R. (2005) The family of *Serratia* type pore forming toxins. *Current Protein & Peptide Science* 6, 313-25.

Hondorp, E. R. & Matthews, R. G. (2004) Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. *PLoS Biology* 2, e336.

Hope, R., Mushtaq, S., James, D., Pllana, T., Warner, M. & Livermore, D. M. (2010) Tigecycline activity: low resistance rates but problematic disc breakpoints revealed by a multicentre sentinel survey in the UK. *Journal of Antimicrobial Chemotherapy* 65, 2602-9.

Hopfe, M., Dahlmanns, T. & Henrich, B. (2011) In *Mycoplasma hominis* the OppA-mediated cytoadhesion depends on its ATPase activity. *BMC Microbiology* 11, 185.

Hornsey, M., Ellington, M. J., Doumith, M., Thomas, C. P., Gordon, N. C., Wareham, D. W., Quinn, J., Lolans, K., Livermore, D. M. & Woodford, N. (2010a) AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 65, 1589-93.

Hornsey, M., Ellington, M. J., Doumith, M., Scott, G., Livermore, D. M. & Woodford, N. (2010b). Emergence of AcrAB-mediated tigecycline resistance in a clinical isolate of *Enterobacter cloacae* during ciprofloxacin treatment. *International Journal of Antimicrobial Agents* 35, 478-81.

Hornsey, M., Ellington, M. J., Doumith, M., Hudson, S., Livermore, D. M. & Woodford, N. (2010c) Tigecycline resistance in *Serratia marcescens* associated with up-regulation of the SdeXY-HasF efflux system also active against ciprofloxacin and cefpirome. *Journal of Antimicrobial Chemotherapy* 65, 479-82.

Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., Underwood, A., Gaulton, T., Thomas, C. P., Doumith, M., Livermore, D. M. & Woodford, N. (2011) Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *Journal of Antimicrobial Chemotherapy* 66, 1499-503.

Hsu, L. Y., Tan, T. Y., Tam, V. H., Kwa, A., Fisher, D. A., Koh, T. H. & the Network for antimicrobial resistance surveillance (Singapore) (2010). Surveillance and correlation of antibiotic prescription and resistance of Gram-negative bacteria in Singaporean hospitals. *Antimicrobial Agents and Chemotherapy* 54, 1173-1178.

Hu, Q., Noll, R. J., Li, H., Makarov, A. & Cooks, R. G. (2005) The Orbitrap: a new mass spectrometer. *Journal of Mass Spectrometry* 40, 430-443.

Huang, Y., Smith, B. S., Chen, L. X., Baxter, R. H. G. & Deisenhofer, J. (2009) Insights into pilus assembly and secretion from the structure and functional characterization of usher PapC. *Proceedings of the National Academy of Sciences of the United States of America* 106, 7403-7.

Huang, Y. H., Lee, Y. L. & Huang, C. Y. (2011) Characterization of a single-stranded DNA binding protein from *Salmonella enterica* serovar Typhimurium LT2. *Protein Journal* 30, 102-108.

Humphries, R. M., Griener, T. P., Vogt, S. L., Mulvey, G. L., Donnenberg, M. S., Kitov, P. I., Surette, M. & Armstrong, G. D. (2011).N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. *Molecular Microbiology* 76, 1111-1126.

Istivan, T. S. & Coloe, P. J. (2006) Phospholipase A in Gram-negative bacteria and its role in pathogenesis. *Microbiology* 152, 1263-74.

Jacobsen, S. M., Lane, M. C., Harro, J. M., Shirtliff, M. E. & Mobley, H. L. T. (2008) The high-affinity phosphate transporter Pst is a virulence factor for *Proteus mirabilis* during complicated urinary tract infection. *FEMS Immunology and Medical Microbiology* 52, 180-93.

Jacoby, G. A. (2005) Mechanisms of resistance to quinolones. *Clinical Infectious Diseases* 41, S120-6.

Jacoby, G. A., Mills, D. M. & Chow, N. (2004) Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* 48, 3203-3206.

Jain, S. & Goldberg, M. B. (2007) Requirement for YaeT in the outer membrane assembly of autotransporter proteins. *Journal of Bacteriology* 189, 5393-8.

Jeon, B., Wang, Y., Hao, H., Barton, Y.W. & Zhang, Q. (2011) Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni*. *Journal of Antimicrobial Chemotherapy* 66, 79-85.

Jeon, J., Kim, H., Yun, J., Ryu, S., Groisman, E. A. & Shin, D. (2008) RstA-promoted expression of the ferrous iron transporter FeoB under iron-replete conditions enhances Fur activity in *Salmonella enterica*. *Journal of Bacteriology* 190, 7326-34.

Johnson, A. P. (2011) Methicillin-resistant *Staphylococcus aureus*: the European Landscape. *Journal of Antimicrobial Chemotherapy* 66, iv43-iv48

Jones, C. H. A. L., Bolken, T. C., Jones, K. F. & Zeller, G. O. (2001) Conserved DegP protease in Gram-positive bacteria is essential for thermal and oxidative tolerance and full virulence in *Streptococcus pyogenes*. *Infection and Immunity* 69, 5538-5545.

Jung, K., Gannoun, A., Sitek, B., Helmut, E. M., Stuhler, K. & Bochum, R. (2005) Analysis of dynamic protein expression data. *Revstat* 3, 99-111.

Kanjee, U., Gutsche, I., Alexopoulos, E., Zhao, B., Bakkouri, M. E., Thibault, G., Liu, K., Ramachandran, S., Snider, J., Pai, E. F. & Houry, W.A. (2011) Linkage between the bacterial acid stress and stringent responses: the structure of the inducible lysine decarboxylase. *The EMBO Journal* 30, 931-944.

Karageorgopoulos, D. E., Kelesidis, T., Kelesidis, I. & Falagas, M. E. (2008) Tigecycline for the treatment of multidrug-resistant (including carbapenem-resistant) *Acinetobacter* infections: a review of the scientific evidence. *Journal of Antimicrobial Chemotherapy* 62, 45-55.

Karisik, E., Ellington, M. J., Pike, R., Warren, R. E., Livermore, D. M. & Woodford, N. (2006) Molecular characterization of plasmids encoding CTX-M-15 β-lactamases from *Escherichia coli* strains in the United Kingdom. *Journal of Antimicrobial Chemotherapy* 58, 665-8.

Karthikeyan, K., Thirunarayan, M. A. & Krishnan, P. (2010) Coexistence of *bla*OXA-23 with *bla*NDM-1 and armA in clinical isolates of *Acinetobacter baumannii* from India. *Journal of Antimicrobial Chemotherapy* 65, 2253-4.

Kattan, J. N., Villegas, M. V. & Quinn, J. P. (2008) New developments in carbapenems. *Clinical Microbiology and Infection* 14, 1102-11.

Keeney, D., Ruzin, A. & Bradford, P. A. (2007) RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. *Microbial Drug Resistance* 13, 1-6.

Kelesidis, T., Karageorgopoulos, D. E., Kelesidis, I. & Falagas, M. E. (2008) Tigecycline for the treatment of multidrug-resistant *Enterobacteriaceae*: a systematic review of the evidence from microbiological and clinical studies. *Journal of Antimicrobial Chemotherapy* 62, 895-904.

Kim, Y. H., Lee, Y., Kim, S., Yeom, J., Yeom, S., Kim, B. S., Oh, S., Park, S., Jeon, C. O. & Park, W. (2006) The role of periplasmic antioxidant enzymes (superoxide dismutase and thiol peroxidase) of the Shiga toxin-producing *Escherichia coli* O157:H7 in the formation of biofilms. *Proteomics* 6, 6181-6193.

Klein, K. A., Fukuto, H. S., Pelletier, M., Romanov, G., Grabenstein, J. P., Palmer, L. E., Ernst, R. & Bliska, J. B. (2012) A transposon site hybridization screen identifies galU and wecBC as important for survival of *Yersinia pestis* in murine macrophages. *Journal of Bacteriology* 194, 653-662.

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B. & Fridkin, S. C. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Journal of the American Medical Association* 298, 1763-1771.

Kneidinger, B., Marolda, C., Graninger, M., Zamyatina, A., Mcarthur, F., Kosma, P., Valvano, M. A. & Messner, P. (2002) Biosynthesis pathway of ADP-L-glycero-β-D-manno-heptose in *Escherichia coli*. *Journal of Bacteriology* 184, 363-369.

Kneller, R. (2010) The importance of new companies for drug discovery: origins of a decade of new drugs. *Nature Reviews Drug Discovery* 9, 867-882.

Kolker, E., Makarova, K. S., Shabalina, S., Picone, A. F., Purvine, S., Holzman, T., Cherny, T., et al. (2004). Identification and functional analysis of 'hypothetical' genes expressed in *Haemophilus influenzae*. *Nucleic Acids Research* 32, 2353-2361.

Korkhov, V. M. & Tate, C. G. (2008) Electron crystallography reveals plasticity within the drug binding site of the small multidrug transporter EmrE. *Journal of Molecular Biology* 377, 1094-1103.

Koskenkorva-frank, T. S. & Kalmo, P. T. (2003) Induction of *Pseudomonas aeruginosa* fhp and fhpR by reactive oxygen species. *Canadian Journal of Microbiology* 55, 657-663.

Krachler, A. M., Sharma, A., Cauldwell, A., Papadakos, G. & Kleanthous, C. (2010). TolA modulates the oligomeric status of YbgF in the bacterial periplasm. *Journal of Molecular Biology* 403, 270-285.

Kraus, C. N. (2008). Low hanging fruit in infectious disease drug development. *Current Opinion in Microbiology* 11, 434-438.

Kristan, K., Sova, M., Gobec, S., & Pre, A. (2009) Novel inhibitors of β-ketoacyl-ACP reductase from *Escherichia coli*. *Chemico-Biological Interactions* 178, 310-316.

Kristian, S. A., Du, M. & Strijp, J. A. G. V. (2003) MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. *Infection and Immunity* 71, 546-549.

Kuczynska-Wisnik, D., Matuszewska, E. & Laskowska, E. (2010) *Escherichia coli* heat-shock proteins IbpA and IbpB affect biofilm formation by influencing the level of extracellular indole. *Microbiology* 156, 148-157.

Kumar, S., Smith, K. P., Floyd, J. L. & Varela, M. F. (2011) Cloning and molecular analysis of a mannitol operon of phosphoenolpyruvate-dependent phosphotransferase (PTS) type from *Vibrio cholerae* O395. *Archives of Microbiology* 193, 201-208.

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C. G., Irfan, S., Krishnan, P., Kumar, A. V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D. L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J. B., Sharma, M., Sheridan, E., Thirunarayan, M. A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D. M. & Woodford, N. (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infectious Diseases* 10, 597-602.

Kurupati, P., Teh, B. K., Kumarasinghe, G. & Poh, C. L. (2006) Identification of vaccine candidate antigens of an ESBL-producing *Klebsiella pneumoniae* clinical strain by immunoproteome analysis. *Proteomics* 6, 836-44.

Kurz, C. L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G. P. F., Uh, M., et al. (2003). Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *The EMBO journal* 22, 1451-60.

Lai, C. C., Wang, C. Y., Chu, C. C., Tan, C. K., Lu, C. L., Lee, Y. C., Huang, Y. T. Lee, P. I. & Hsueh, P. R. (2011) Correlation between antibiotic consumption and resistance of Gram-negative bacteria causing healthcare-associated infections at a university hospital in Taiwan from 2000 to 2009. *Journal of Antimicrobial Chemotherapy* 66, 1374-1382.

Lartigue, M.-F., Poirel, L., Poyart, C., Réglier-Poupet, H. & Nordmann, P. (2007) Ertapenem resistance of *Escherichia coli*. *Emerging Infectious Diseases* 13, 315-7.

Laurent, J., Vogel, C., Kwon, T., Craig, S. A., Boutz, D. R., Huse, H. K., Nozue, K., Walia, H., Whiteley, M., Ronald, P. C. & Marcotte, E. M. (2011) Protein abundances are more conserved than mRNA abundances across diverse taxa. *Proteomics* 10, 4209-4212.

Law, R. J., Hamlin, J. N. R., Sivro, A., Mccorrister, S. J., Cardama, G. A. & Cardona, S. T. (2008). A functional phenylacetic acid catabolic pathway is required for full pathogenicity of

Burkholderia cenocepacia in the *Caenorhabditis elegans* host model. *Journal of Bacteriology* 190, 7209-7218.

Lear, J. C., Maillard, J., Dettmar, P. W., Goddard, P. A., & Russell, A. D. (2002). Chloroxylenol- and triclosan-tolerant bacteria from industrial sources. *Journal of Industrial Microbiology and Biotechnology* 29, 238-242.

Lee, N., Yuen, K. Y. & Kumana, C. R. (2003). Clinical role of β-lactam / β-lactamase inhibitor combinations. *Drugs* 63, 1511-1524.

Leroux, P., Gredt, M., Leroch, M. & Walker, A. S. (2010) Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Applied and Environmental Microbiology* 76, 6615-6630.

Letoffe, S., Delepelaire, P. & Wandersman, C. (2006) The housekeeping dipeptide permease is the *Escherichia coli* heme transporter and functions with two optional peptide binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* 103, 12891-12896.

Levy, S. B. & Marshall, B. (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* 10, S122-9.

Li, Z., Adams, R. M., Chourey, K., Hurst, G. B. & Hettich, R. L. (2012) Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. *Journal of Proteome Research* 11, 1582-1590.

Liaqat, I. & Thomas, R. (2010) Isolate-specific effects of patulin, penicillic acid and EDTA on biofilm formation and growth of dental unit water line biofilm isolates. *Current Microbiology* 61, 148-156.

Limsuwun, K. & Jones, P. G. (2000) Spermidine acetyltransferase is required to prevent spermidine toxicity at low temperatures in *Escherichia coli*. *Journal of Bacteriology* 182, 5373-5380.

Liu, C., Wang, N., Lee, C., Weng, L. & Tseng, H. (2004) Nosocomial and community-acquired *Enterobacter cloacae* bloodstream infection: risk factors for and prevalence of SHV-12 in multiresistant isolates in a medical centre. *Journal of Hospital Infection* 58, 63-77.

Liu, G., Zhou, J., Fu, Q. S. & Wang, J. (2009). The *Escherichia coli* azoreductase AzoR is involved in resistance to thiol-specific stress caused by electrophilic quinones. *Journal of Bacteriology* 191, 6394-6400.

Liu, Y., Bauer, S. C. & Imlay, J. A. (2011) The YaaA protein of the *Escherichia coli* OxyR regulon lessens hydrogen peroxide toxicity by diminishing the amount of intracellular unincorporated iron. *Journal of Bacteriology* 193, 2186-2196.

Livermore, D M. (2004). The need for new antibiotics. *Clinical Microbiology and Infection* 10, S1-9.

Livermore, D M. (2008). Defining an extended-spectrum beta-lactamase. *Clinical Microbiology and Infection* 14, S3-10.

Livermore, D M, Winstanley, T. G. & Shannon, K. P. (2001) Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *Journal of Antimicrobial Chemotherapy* 48, S87-102.

Livermore, D. M., Hope, R., Brick, G., Lillie, M. & Reynolds, R. (2008) Non-susceptibility trends among Enterobacteriaceae from bacteraemias in the UK and Ireland, 2001-06. *Journal of Antimicrobial Chemotherapy* 62, ii41-54.

Livermore, D.M. (2005) Tigecycline: what is it, and where should it be used? *Journal of Antimicrobial Chemotherapy* 56, 611-4.

Livermore, D. M. (2009) Has the era of untreatable infections arrived? *Journal of Antimicrobial Chemotherapy* 64, i29-36.

Livermore, D.M. & Woodford, N. (2006). The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. *Trends in Microbiology* 14, 413-20.

Luo, P. & Morrison, D. A. (2003) Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in *Streptococcus pneumoniae*. *Journal of Bacteriology* 185, 349-358.

Lutkenhaus, J. (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annual Review of Biochemistry* 76, 539-62.

Macgowan, A. P. & Wise, R. (2001) Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *Journal of Antimicrobial Chemotherapy* 48, 17-28.

Macrae, I. J. & Doudna, J. A. (2007) Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Current Opinion in Structural Biology* 17, 138-145.

Madeira, A., Santos, P. M., Coutinho, C. P., Pinto-de-Oliveira, A. & Sá-Correia, I. (2011) Quantitative proteomics (2-D DIGE) reveals molecular strategies employed by *Burkholderia cenocepacia* to adapt to the airways of cystic fibrosis patients under antimicrobial therapy. *Proteomics* 11, 1313-28.

Magiorakos, A., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T. & Monnet, D. L. (2011) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* 18, 268-281.

Mahlen, S. D. (2011) *Serratia* infections: from military experiments to current practice. *Clinical Microbiology Reviews* 24, 755-791.

Mammeri, H., Nordmann, P., Berkani, A. & Eb, F. (2008) Contribution of extended-spectrum AmpC (ESAC) β-lactamases to carbapenem resistance in Escherichia coli. *FEMS Microbiology Letters* 282, 238-40.

Maragakis, L. L. & Perl, T. M. (2008) *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. *Clinical Infectious Diseases* 46, 1254-63.

Maragakis, L. L., Winkler, A., Tucker, M. G., Cosgrove, S. E., Ross, T., Lawson, E., Carroll, K. C. & Perl, T. M. (2012) Outbreak of multidrug-resistant *Serratia marcescens* infection in a neonatal intensive care unit. *Infection Control and Hospital Epidemiology* 29, 418-423.

March, R. E. (2009) Quadrupole ion traps. *Mass Spectrometry Reviews* 28, 961-989.

Margus, T., Remm, M. & Tenson, T. (2007) Phylogenetic distribution of translational GTPases in bacteria. *BMC Genomics* 8, 1-18.

Martinez, J. L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution* 157, 2893-2902.

Martinez, J. L., Sánchez, M. B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A. & Alvarez-Ortega, C. (2009) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiology Reviews* 33, 430-49.

Martins, A., Spengler, G., Rodrigues, L., Viveiros, M., Ramos, J., Martins, M., Couto, I., Fanning, S., Pages, J. M., Bolla, J. M., Molnar, J. & Amaral, L. (2009) pH Modulation of efflux pump activity of multi-drug resistant *Escherichia coli*: protection during its passage and eventual colonization of the colon. *PloS one* 4, e6656.

Martínez-Martínez, L. (2008). Extended-spectrum beta-lactamases and the permeability barrier. *Clinical Microbiology and Infection* 14, 82-9.

Marzoa, J., Sánchez, S., Costoya, L., Diéguez-casal, E., Freixeiro, P., Brookes, C., Allen, L., Taylor, S., Gorringe, A. R., Ferreiros, C. M. & Criado, M. T. (2012) Induction of immune responses by purified outer membrane protein complexes from *Neisseria meningitidis*. *Vaccine* 30, 2387-2395.

Masi, M., Pagès, J.-M. & Pradel, E. (2006) Production of the cryptic EefABC efflux pump in *Enterobacter aerogenes* chloramphenicol-resistant mutants. *Journal of Antimicrobial Chemotherapy* 57, 1223-6.

Matsumura, K., Furukawa, S., Ogihara, H. & Morinaga, Y. (2011) Roles of multidrug efflux pumps on the biofilm formation of Escherichia coli K-12. *Biocontrol Science* 16, 69-72

Matsuo, T., Chen, J., Minato, Y., Ogawa, W., Mizushima, T., Kuroda, T. & Tsuchiya, T. (2008). SmdAB, a heterodimeric ABC-Type multidrug efflux pump, in *Serratia marcescens*. *Journal of Bacteriology* 190, 648-654.

Mcmurry, L., Petrucci, R. E. & Levy, S. B. (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 77, 3974-3977.

Mera, P., & Escalante-Semerena, J. (2011) Multiple roles of ATP:cob(I)alamin adenosyltransferases in the conversion of B12 to coenzyme B12. *Applied Microbiology and Biotechnology* 88, 41-48.

Miller, E. N., Jarboe, L. R., Yomano, L. P., York, S. W., Shanmugam, K. T. & Ingram, L. O. (2009) Silencing of NADPH-dependent oxidoreductase genes (yqhD and dkgA) in furfural-resistant ethanologenic *Escherichia coli*. *Applied and Environmental Microbiology* 75, 4315-4323.

Miller, J. H., Funchain, P., Clendenin, W., Huang, T., Nguyen, A., Wolff, E., Yeung, A., et al. (2002). *Escherichia coli* strains (ndk) lacking nucleoside diphosphate kinase are powerful mutators for base substitutions and frameshifts in mismatch-repair-deficient strains. *Genetics* 162, 5-13.

Misra, R. V., Ahmod, N. Z., Parker, R., Fang, M., Shah, H. & Gharbia, S. (2012) Developing an integrated proteo-genomic approach for the characterisation of biomarkers for the identification of *Bacillus anthracis*. *Journal of Microbiological Methods* 88, 237-247.

Mitsuhashi, S., Harada, K. & Hashimoto, H. (1960) Multiple resistance of enteric bacteria and transmission of drug-resistance to other strains by mixed cultivation. *Japanese Journal of Experimental Medicine* 30, 179-184.

Mittal, R., Wang, Y., Hunter, C. J., Gonzalez-Gomez, I. & Prasadarao, N. V. (2009) Brain damage in newborn rat model of meningitis by *Enterobacter sakazakii*: a role for outer membrane protein A. *Laboratory investigation* 89, 263-77.

Mizanur, R. M. & Pohl, N. L. (2008). Bacterial CMP-sialic acid synthetases: production, properties, and applications. *Applied Microbiology and Biotechnology* 80, 757-65.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D. F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St.Michael, F., Cox, A. D., Adler, B., Nation, R. L., Li, J. & Boyce, J. D. (2010) Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy* 54, 4971-7.

Morales, C. A., Porwollik, S., Frye, J. G., Kinde, H., Mcclelland, M. & Guard-bouldin, J. (2005) Correlation of phenotype with the genotype of egg-contaminating *Salmonella enterica* serovar enteritidis. *Applied and Environmental Microbiology* 71, 4388-4399.

Moreno-paz, M., Gómez, M. J., Arcas, A. & Parro, V. (2010) Environmental transcriptome analysis reveals physiological differences between biofilm and planktonic modes of life of the iron oxidizing bacteria *Leptospirillum* spp. in their natural microbial community. *BMC Genomics* 11, 1-14

Mouslim, C., Cano, D., Flores, A. & Casadesus, J. (2000). Regulation of septation: a novel role for SerC/PdxF in *Salmonella*? *Molecular And General Genetics* 264, 184-192.

Nagy, E. & Dowzicky, M. J. (2010) In vitro activity of tigecycline and comparators against a European compilation of anaerobes collected as part of the Tigecycline Evaluation and Surveillance Trial (TEST). *Scandanavian Journal of Infectious Diseases* 42, 33-38.

Narita, S. I. & Tokuda, H. (2011) Overexpression of LolCDE allows deletion of the *Escherichia coli* gene encoding apolipoprotein N-acetyltransferase. *Journal of Bacteriology* 193, 4832-4840.

Nesper, J., Lauriano, C. M., Klose, K. E., Kapfhammer, D., Kraiß, A. & Reidl, J. (2001) Characterization of *Vibrio cholerae* O1 El Tor galU and galE Mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infection and Immunity* 69, 435-445.

Newton, S. M., Trinh, V., Pi, H. & Klebba, P. E. (2010) Direct measurements of the outer membrane stage of ferric enterobactin transport. *Journal of Biological Chemistry* 285, 17488 -17497.

Nikaido, H. (2009) Multidrug resistance in bacteria. *Annual Review of Biochemistry* 78, 119-46.

Nordmann, P., Cuzon, G. & Naas, T. (2009) The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *The Lancet Infectious Diseases* 9, 228-236.

Nouwen, N., van der Laan, M. & Driessen, A. J. (2001). SecDFyajC is not required for the maintenance of the proton motive force. *FEBS letters* 508, 103-6.

Oku, Y., Kurokawa, K., Ichihashi, N. & Sekimizu, K. (2004) Characterization of the *Staphylococcus aureus* mprF gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* 150, 45-51.

O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* 250, 4007-4021.

Palomino, C., Marín, E. & Luis, A. (2011) The fimbrial usher FimD follows the SurA-BamB pathway for its assembly in the outer membrane of *Escherichia coli*. *Journal of Bacteriology* 193, 5222-5230.

Pancholi, V. & Fischetti, V. A. (1998) α-Enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic *Streptococci*. *Journal of Biological Chemistry* 273, 14503-15.

Park, M. R., Lee, E. G., Kim, Y. H., Jung, T. S., Shin, Y. S., Shin, G. W., Cha, H. G. & Kim, G. S. (2003) Reference map of soluble proteins from *Salmonella enteritidis* by two-dimensional electrophoresis. *Journal of Veterinary Science* 4, 143-149.

Park, S. H., Lee, H. W. & Cao, W. (2010) Microbial pathogenesis screening of nitrosative stress resistance genes in *Coxiella burnetii*: involvement of nucleotide excision repair. *Microbial Pathogenesis* 49, 323-329.

Patching, S. G., Baldwin, S. A., Baldwin, A. D., Young, J. D., Gallagher, M. P., Henderson, J. F. & Herbert, R. B. (2005) The nucleoside transport proteins, NupC and NupG, from *Escherichia coli*: specific structural motifs necessary for the binding of ligands. *Organic and Biomolecular Chemistry* 3, 462-470.

Paterson, D. L. (2006). Resistance in Gram-negative bacteria: Enterobacteriaceae. *American Journal of Infection Control* 35, S20-8.

Peirce, M. J., Wait, R., Begum, S., Saklatvala, J. & Cope, A. P. (2004) Expression profiling of lymphocyte plasma membrane proteins. *Molecular & Cellular Proteomics* 3, 56-65.

Peleg, A. Y., Adams, J. & Paterson, D. L. (2007) Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* 51, 2065-9.

Persky, N. S., Ferullo, D. J., Cooper, D. L., Moore, H. R. & Lovett, S. T. (2009) The ObgE/CgtA GTPase influences the stringent response to amino acid starvation in *Escherichia coli*. *Molecular Microbiology* 73, 253-266.

Phadtare, S. & Severinov, K. (2009) Comparative analysis of changes in gene expression due to RNA melting activities of translation initiation factor IF1 and a cold shock protein of the CspA family. *Genes to Cells* 14, 1227-1239.

Pichler, P., Köcher, T., Holzmann, J., Mazanek, M., Taus, T., Ammerer, G. & Mechtler, K. (2010) Peptide labeling with isobaric tags yields higher identification rates using iTRAQ 4-plex compared to TMT 6-plex and iTRAQ 8-plex on LTQ Orbitrap. *Analytical Chemistry* 82, 6549-58.

Piddock, L. J. V. (2002) Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS Microbiology Reviews* 26, 3-16.

Piddock, L. J. V. (2006) Multidrug-resistance efflux pumps - not just for resistance. *Nature Reviews Microbiology* 4, 629-36.

Pilonieta, M. C., Erickson, K. D., Ernst, R. K. & Detweiler, C. S. (2009) A protein important for antimicrobial peptide resistance, YdeI/OmdA, is in the periplasm and interacts with OmpD/NmpC. *Journal of Bacteriology* 191, 7243-52.

Pitout, J. D. D., Nordmann, P., Laupland, K. B. & Poirel, L. (2005) Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. *Journal of Antimicrobial Chemotherapy* 56, 52-59.

Plessis, D. J. F., Nouwen, N. & Driessen, A. J. M. (2011) The Sec translocase. *Biochimica et Biophysica Acta* 1808, 851-865.

Poggio, S., Takacs, C. N., Vollmer, W. & Jacobs-Wagner, C. (2011) A protein critical for cell constriction in the Gram-negative bacterium *Caulobacter crescentus* localizes at the division site through its peptidoglycan-binding LysM domains. *Molecular Microbiology* 77, 74-89.

Poirel, L., Gniadkowski, M. & Nordmann, P. (2002) Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. *Journal of Antimicrobial Chemotherapy* 50, 1031-1034.

Poirel, L., Cattoir, V. & Nordmann, P. (2012) Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. *Frontiers in Microbiology* 3, 24.

Poirel, L., Heritier, C., Spicq, C. & Nordmann, P. (2004) In vivo acquisition of high-level resistance to imipenem in *Escherichia coli*. *Journal of Clinical Microbiology* 42, 3831-3833.

Porteus, B., Kocharunchitt, C., Nilsson, R. E., Ross, T. & Bowman, J. P. (2011) Utility of gel-free, label-free shotgun proteomics approaches to investigate microorganisms. *Applied Microbiology and Biotechnology* 90, 407-416.

Pradel, N., Delmas, J., Wu, L. F., Santini, C. L., & Bonnet, R. (2009) Sec- and Tat-dependent translocation of β-lactamases across the *Escherichia coli* inner membrane. *Antimicrobial Agents and Chemotherapy* 53, 242-248.

Puorger, C., Vetsch, M., Wider, G. & Glockshuber, R. (2011) Structure, folding and stability of FimA, the main structural subunit of type 1 pili from uropathogenic *Escherichia coli* strains. *Journal of Molecular Biology* 412, 520-535.

Pérez, A., Canle, D., Latasa, C., Poza, M., Beceiro, A., Tomás, M. D. M., Fernández, A., Mallo, S., Perez, S., Molina, F., Villanueva, R., Lasa, I. & Bou, G. (2007) Cloning, nucleotide sequencing, and analysis of the AcrAB-TolC efflux pump of *Enterobacter cloacae* and determination of its involvement in antibiotic resistance in a clinical isolate. *Antimicrobial Agents and Chemotherapy* 51, 3247-53.

Pérez, A., Poza, M., Fernández, A., Fernández, C., Mallo, S., Merino, M., Rumbo-Feal, S., Cabral, M. P. & Bou, G. (2012) Involvement of the AcrAB-TolC efflux pump in resistance, fitness and virulence of *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* 56, 2084-90.

Queenan, A. M. & Bush, K. (2007) Carbapenemases: the versatile β-lactamases. *Clinical Microbiology Reviews* 20, 440-58.

Qureshi, Z. A., Paterson, D. L., Potoski, B. A., Kilayko, M. C., Sandovsky, G., Sordillo, E., Polsky, B., Adams-Haduch, J. M. & Doi, Y. (2012) Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: superiority of combination antimicrobial regimens. *Antimicrobial Agents and Chemotherapy* 56, 2108-13.

Ramirez, M. S. & Tolmasky, M. E. (2011) Aminoglycoside modifying enzymes. *Drug Resistance Updates* 13, 151-171.

Randall, L. P. & Woodward, M. J. (2002) The multiple antibiotic resistance (mar) locus and its significance. *Research in Veterinary Science* 72, 87-93.

Reyes-lamothe, R., Sherratt, D. J. & Leake, M. C. (2010) Stoichiometry and architecture of active DNA replication machinery in *Escherichia coli*. *Science* 328, 498-501.

Rezzonico, F. & Duffy, B. (2008) Lack of genomic evidence of AI-2 receptors suggests a non-quorum sensing role for luxS in most bacteria. *BMC microbiology* 8, 154.

Riley, M. A., Pinou, T., Wertz, J. E., Tan, Y. & Valletta, C. M. (2001) Molecular Characterization of the Klebicin B Plasmid of *Klebsiella pneumoniae*. *Plasmid* 45, 209-221.

Rippa, V., Cirulli, C., Palo, B. D., Doti, N., Amoresano, A. & Duilio, A. (2010) The ribosomal protein L2 Interacts with the RNA Polymerase alpha subunit and acts as a transcription modulator in *Escherichia coli*. *Journal of Bacteriology* 192, 1882-1889.

Rossolini, G. M. & Mantengoli, E. (2008) Antimicrobial resistance in Europe and its potential impact on empirical therapy. *Clinical Microbiology and Infection* 14, 2-8.

Rossouw, F. T. & Rowbury, R. J. (1984) Effects of the resistance plasmid R124 on the level of the OmpF outer membrane protein and on the response of Escherichia coli to environmental agents. *Journal of Applied Bacteriology* 56, 63-79.

Roucourt, B., Minnebo, N., Augustijns, P., Hertveldt, K., Volckaert, G. & Lavigne, R. (2009) Biochemical characterization of malate synthase G of *P. aeruginosa*. *BMC Biochemistry*, 10, 20.

Ruan, L., Pleitner, A., Gänzle, M. G. & McMullen, L. M. (2011) Solute transport proteins and the outer membrane protein NmpC contribute to heat resistance of *Escherichia coli* AW1.7. *Applied and Environmental Microbiology* 77, 2961-7.

Russell, A. D. (1997) Plasmids and bacterial resistance to biocides. *Journal of Applied Microbiology* 82, 155-165.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., Macdonald, U., Schultz, L. W., Umland, T. C. & Campagnari, A. A. (2010) The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and Immunity* 78, 3993-4000.

Ruzin, A., Keeney, D. & Bradford, P. A. (2007) AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *Journal of Antimicrobial Chemotherapy* 59, 1001-4.

Ruzin, A., Visalli, M. A., Keeney, D. & Bradford, P. A. (2005) Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* 49, 1017-1022.

Sabbagh, C., Lepage, C., Mcclelland, M. & Daigle, F. (2012) Selection of *Salmonella enterica* serovar Typhi genes involved during interaction with human macrophages by screening of a transposon mutant library. *PloS one* 7, e36643.

Sanders, W. E. & Sanders, C. C. (1997) *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. *Clinical Microbiology Reviews* 10, 220-41.

Santiago, B., Macgilvray, M., Faustoferri, R.C. & Quivey Jr, R. G. (2012) The branched-chain amino acid aminotransferase encoded by *ilvE* is involved in acid tolerance in *Streptococcus mutans*. *Journal of Bacteriology* 194, 2010-19.

Sardis, M. F. & Economou, A. (2010) SecA: a tale of two protomers. *Molecular Microbiology* 76, 1070-1081.

Saxena, S. & Gowrishankar, J. (2011). Compromised factor-dependent transcription termination in a nusA mutant of *Escherichia coli*: spectrum of termination efficiencies generated by perturbations of Rho , NusG , NusA , and H-NS family proteins. *Journal of Bacteriology* 193, 3842-3850.

Schmidt, F., Scharf, S. S., Hildebrandt, P., Burian, M., Bernhardt, J., Dhople, V., Kalinka, J., Gutjahr, M., Hammer, E. & Volker, U. (2010) Time-resolved quantitative proteome profiling of host – pathogen interactions: the response of *Staphylococcus aureus* RN1HG to internalisation by human airway epithelial cells. *Proteomics* 10, 2801-2811.

Schnell, R., Oehlmann, W., Sandalova, T., Braun, Y., Huck, C., Singh, M. & Schneider, G. (2012) Tetrahydrodipicolinate N-succinyltransferase and dihydrodipicolinate synthase from *Pseudomonas aeruginosa*: structure analysis and gene deletion. *PLoS one* 7, e31133.

Seidler, J., Zinn, N., Boehm, M. E. & Lehmann, W. D. (2010) De novo sequencing of peptides by MS/MS. *Proteomics* 10, 634-649.

Sha, J., Erova, T. E., Alyea, R. A, Wang, S., Olano, J. P., Pancholi, V., & Chopra, A. K. (2009). Surface-expressed enolase contributes to the pathogenesis of clinical isolate SSU of Aeromonas hydrophila. *Journal of Bacteriology* 191, 3095-107.

Sharma, J., Ray, P., Sharma, M. & Ghosh, S. (2010) Comparison of isoelectric focusing and polymerase chain reaction for the detection of β-lactamases. *Indian Journal of Medical Microbiology* 28, 376-380.

Sheldon, A. T. (2005) Antiseptic "resistance": real or perceived threat? *Clinical Infectious Diseases* 40, 1650-1656.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H. & Mann, M. (1996) Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two-dimensional gels. *Proceedings of the National Academy of Sciences of the United States of America* 93, 14440-14445.

Shin, J. H., Lee, H. W., Kim, S. M. & Kim, J. (2009). Proteomic analysis of *Acinetobacter baumannii* in biofilm and planktonic growth mode. *Journal of Microbiology* 47, 728-35.

Shirazi-Beechey, S., & Knowles, C. (1984). Serine hydroxymethyltransferase and glycine cleavage enzyme from the cyanogenic bacterium *Chromobacterium violaceum*. *Journal of General Microbiology* 130, 521-525.

Shoji, M., Ratnayake, D. B., Shi, Y., Kadowaki, T., Yamamoto, K., Yoshimura, F., Akamine, A., Curtis, M. A. & Nakayama, K. (2002) Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. *Microbiology* 148, 1183-1191.

Simmons, K. J., Chopra, I. & Fishwick, C. W. G. (2010) Structure-based discovery of antibacterial drugs. *Nature Reviews Microbiology* 8, 501-10.

Simpson, S. A., Butler, C. C., Hood, K., Cohen, D., Dunstan, F., Evans, M. R., Rollnick, S., Moore, L., Hare, M., Bekkers, M. J., Evans, J. & STAR study team. (2009) Stemming the Tide of Antibiotic Resistance (STAR): A protocol for a trial of a complex intervention addressing the 'why' and 'how' of appropriate antibiotic prescribing in general practice. *BMC Family Practice* 10, 20.

Siroy, A., Cosette, P., Seyer, D., Lemaître-Guillier, C., Vallenet, D., Van Dorsselaer, A., Boyer-Mariotte, S., Jouenne, T. & De, E. (2006) Global comparison of the membrane subproteomes between a multidrug-resistant *Acinetobacter baumannii* strain and a reference strain. *Journal of Proteome Research* 5, 3385-98.

Sivick, K. E. & Mobley, H. L. T. (2010) Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. *Infection and Immunity* 78, 568-585.

Sköld, O. S. (2001) Resistance to trimethoprim and sulfonamides. *Veterinary Research* 32, 261-273.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C. & de la Cruz, F. (2010) Mobility of plasmids. *Microbiology and Molecular Biology Reviews* 74, 434-52.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M. & Snyder, M. (2007) New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes & Development* 21, 601-614.

Smith, S. G. J., Mahon, V., Lambert, M. A. & Fagan, R. P. (2007) A molecular Swiss army knife: OmpA structure, function and expression. *FEMS microbiology letters* 273, 1-11.

Smither, S. J., Hill, J., van Baar, B. L. M., Hulst, A. G., de Jong, A. L. & Titball, R. W. (2007) Identification of outer membrane proteins of *Yersinia pestis* through biotinylation. *Journal of Microbiological Methods* 68, 26-31.

Soares, N. C., Cabral, M. P., Gayoso, C., Mallo, S., Rodriguez-Velo, P., Fernández-Moreira, E. & Bou, G. (2010) Associating growth-phase-related changes in the proteome of *Acinetobacter baumannii* with increased resistance to oxidative stress. *Journal of Proteome Research* 9, 1951-64.

Soares, N. C., Cabral, M. P., Parreira, J. R., Gayoso, C., Barba, M. J. & Bou, G. (2009) 2-DE analysis indicates that *Acinetobacter baumannii* displays a robust and versatile metabolism. *Proteome science* 7, 37.

Song, J. S., Jeon, J. H., Lee, J. H., Jeong, S. H., Jeong, B. C., Kim, S. J., Lee, J. H. & Sang, H. L. (2005) Molecular characterisation of TEM-type β-lactamases identified in cold-seep sediments of edison seamount (south of Lihir Island, Papua New Guinea). *Journal of Microbiology* 43, 172-178.

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G. & Edwards, J. E. (2004) Trends in antimicrobial drug development: implications for the future. *Clinical Infectious Diseases* 38, 1279-86.

Stalder, T., Barraud, O., Casellas, M., Dagot, C. & Ploy, M. C. (2012) Integron involvement in environmental spread of antibiotic resistance. *Frontiers in Microbiology* 3, 1-14.

Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N. & Slonczewski, J. L. (2002) pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *Frontiers in Microbiology* 184, 4246-4258.

Sukupolvi, S. & Connor, C. D. O. (1990) TraT lipoprotein, a plasmid-specified mediator of interactions between Gram-negative bacteria and their environment. *Microbiological Reviews* 54, 331-341.

Szabó, D., Silveira, F., Hujer, A. M., Bonomo, R. a, Hujer, K. M., Marsh, J. W., Bethel, C. R., Doi, Y., Deeley, K. & Paterson, D. L. (2006) Outer membrane protein changes and efflux pump

expression together may confer resistance to ertapenem in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* 50, 2833-5.

Taghbalout, A. & Rothfield, L. (2007) RNaseE and the other constituents of the RNA degradosome are components of the bacterial cytoskeleton. *Proceedings of the National Academy of Sciences of the United States of America* 104, 1667-72.

Tanabe, M., Atkins, H. S., Harland, D. N., Elvin, S. J., Stagg, A. J., Mirza, O., Titball, R. W., Byrne, B. & Brown, K. A. (2006) The ABC transporter protein OppA provides protection against experimental *Yersinia pestis* Infection. *Infection and Immunity* 74, 3687-3691.

Tang, Y., Guest, J. R., Artymiuk, P. J. & Green, J. (2005) Switching aconitase B between catalytic and regulatory modes involves iron-dependent dimer formation. *Molecular Microbiology* 56, 1149-1158.

Tang, Y., Guest, J. R., Artymiuk, P. J., Read, R. C. & Green, J. (2004) Post-transcriptional regulation of bacterial motility by aconitase proteins. *Molecular Microbiology* 51, 1817-1826.

Tani, A., Ishige, T., Sakai, Y. & Kato, N. (2002) Two acyl-CoA dehydrogenases of *Acinetobacter* sp. strain M-1 that uses very long-chain n-alkanes. *Journal of Bioscience and Bioengineering* 94, 326-329.

Tenover, F. C. (2006) Mechanisms of antimicrobial resistance in bacteria. *American journal of Infection Control* 34, S3-10.

Thedieck, K., Hain, T., Mohamed, W., Tindall, B. J., Nimtz, M., Chakraborty, T., Wehland, J. & Jansch, L. (2006) The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. *Molecular Microbiology* 62, 1325-39.

Theuretzbacher, U. (2009) Future antibiotics scenarios: is the tide starting to turn? *International Journal of Antimicrobial Agents* 34, 15-20.

Tikhonova, E. B., Dastidar, V., Rybenkov, V. V. & Zgurskaya, H. I. (2009) Kinetic control of TolC recruitment by multidrug efflux complexes. *Proceedings of the National Academy of Sciences of the United States of America* 106, 16416-16421.

Tischer, M., Pradel, G., Ohlsen, K. & Holzgrabe, U. (2012) Quaternary ammonium salts and their antimicrobial potential: targets or nonspecific interactions? *Chemistry and Medicinal Chemistry* 7, 22-31.

Toleman, M. A. & Walsh, T. R. (2011) Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiology Reviews* 35, 912-935.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E. & Actis, L. A. (2003) Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149, 3473-3484.

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. S. & Actis, L. A. (2008) Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology* 154, 3398-409.

Tomazella, G. G., Risberg, K., Mylvaganam, H., Christoffer, P., Thiede, B., Souza, G. A. D. & Wiker, H. G. (2011) Proteomic analysis of a multi-resistant clinical *Escherichia coli* isolate of unknown genomic background. *Journal of Proteomics* 75, 1830-1837.

Towner, K. J. (2009) Acinetobacter: an old friend, but a new enemy. *The Journal of Hospital Infection* 73, 355-63.

Turner, R. J., Taylor, D. E. & Weiner, J. H. (1997) Expression of *Escherichia coli* TehA gives resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps. *Antimicrobial Agents and Chemotherapy* 41, 440-4.

Turnidge, J., Kahlmeter, G. & Kronvall, G. (2006) Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clinical Microbiology Infections* 12, 418-425

Ueta, M., Yoshida, H., Wada, C., Bab, T., Mori, H. & Wada, A. (2005) Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of *Escherichia coli*. *Genes to Cells* 10, 1103-1112.

Van Houdt, R., Givskov, M. & Michiels, C. W. (2007) Quorum sensing in *Serratia*. *FEMS Microbiology Reviews* 31, 407-24.

Varghese, S., Tang, Y. & Imlay, J. A. (2003) Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. *Journal of Bacteriology* 185, 221-230.

Vashist, J., Tiwari, V., Kapil, A. & Rajeswari, M. R. (2010) Quantitative profiling and identification of outer membrane proteins of beta-lactam resistant strain of *Acinetobacter baumannii*. *Journal of Proteome Research* 9, 1121-8.

Vila, J., Martí, S. & Sánchez-Céspedes, J. (2007) Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 59, 1210-5.

Villet, R., Fonvielle, M., Busca, P., Chemama, M., Maillard, A. P., Hugonnet, J.E., Dubost, L., Marie, A., Josseaume, N., Mesnage, S., Mayer, C., Valery, J. M. & Etheve-Quelquejeu, M. (2007) Idiosyncratic features in tRNAs participating in bacterial cell wall synthesis. *Nucleic Acids Research* 35, 6870-6883.

Voelz, A., Müller, A., Gillen, J., Le, C., Dresbach, T., Engelhart, S., Exner, M., Bates, C. J. & Simon, A. (2010) Outbreaks of *Serratia marcescens* in neonatal and pediatric intensive care units: clinical aspects, risk factors and management. *International Journal of Hygiene and Environmental Health* 213, 79-87.

Vogels, M. W., van Balkom, B. W. M., Heck, A. J. R., de Haan, C. A. M., Rottier, P. J. M., Batenburg, J. J., Kaloyanova, D. V. Helms, J. B. (2011) Quantitative proteomic identification of host factors involved in the *Salmonella typhimurium* infection cycle. *Proteomics* 11, 4477-4491.

Volkers, G., Palm, G. J., Weiss, M. S., Wright, G. D. & Hinrichs, W. (2011) Structural basis for a new tetracycline resistance mechanism relying on the TetX monooxygenase. *FEBS Letters* 585, 1061-1066.

World Health Organisation. (2011) The 10 leading causes of death 2008. Retrieved from http://www.who.int/mediacentre/factsheets/fs310_2008.pdf. Last accessed on: 19/09/12

World Health Organisation. (2012) The evolving threat of antimicrobial resistance: Options for action. Retrieved from http://whqlibdoc.who.int/publications/2012/9789241503181_eng.pdf. Last accessed on: 19/09/12

Wagner, A. F., Schultz, S., Bomke, J., Pils, T., Lehmann, W. D. & Knappe, J. (2001) YfiD of *Escherichia coli* and Y06I of bacteriophage T4 as autonomous glycyl radical cofactors

reconstituting the catalytic center of oxygen-fragmented pyruvate formate-lyase. *Biochemical and Biophysical Research Communications* 285, 456-62.

Waksman, S. (1973) History of the word "Antibiotic". *Journal of the History of Medicine and Allied Sciences* 28, 284-286.

Wang, P., Shim, E., Cravatt, B., Jacobsen, R., Schoeniger, J., Kim, A. C., Paetzel, M. & Dalbey, R. E. (2009) The *Escherichia coli* signal peptide peptidase A is a serine-lysine protease with a lysine recruited to the non-conserved amino-terminal domain in the S49 protease family. *Biochemistry* 47, 6361-6369.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W. Harris, R., Williams, K. L. & Humphrey-Smith, I. (1995) Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis* 16, 1090-1094.

Watanabe, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bacteriological Reviews* 27, 87-115.

Weber, A., Kögl, S. A. & Jung, K. (2006) Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. *Journal of Bacteriology* 188, 7165-75.

Webster, D. P., Gaulton, T., Woodford, N., Pike, R., Turton, J., Perry, C. & Bowler, I. C. J. W. (2010) Emergence of carbapenem resistance due to porin loss in an extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strain during meropenem therapy. *International Journal of Antimicrobial Agents* 36, 575-6.

Wiles, T. J., Kulesus, R. R. & Mulvey, M. A. (2008) Origins and virulence mechanisms of uropathogenic *Escherichia coli*. *Experimental and Molecular Pathology* 85, 11-9.

Wise, E. M. & Abou-donia, M. M. (1975) Sulfonamide resistance mechanism in *Escherichia coli*: R plasmids can determine sulfonamide-resistant dihydropteroate synthases. *Proceedings of the National Academy of Sciences of the United States of America* 72, 2621-2625.

Wittmann-Liebold, B., Graack, H.-R. & Pohl, T. (2006) Two-dimensional gel electrophoresis as tool for proteomics studies in combination with protein identification by mass spectrometry. *Proteomics* 6, 4688-703.

Woodford, N, Ward, M. E., Kaufmann, M. E., Turton, J., Fagan, E. J., James, D., Johnson, A. P., Pike, R., Warner, M., Cheasty, T., Pearson, A., Harry, S., Leach, J. B., Loughrey, A., Lowes, J. A., Warren, R. E. Livermore, D. M. (2004) Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. *Journal of Antimicrobial Chemotherapy* 54, 735-43.

Woodford, N., Carattoli, A., Karisik, E., Underwood, A., Ellington, M. J. & Livermore, D. M. (2009) Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrobial Agents and Chemotherapy* 53, 4472-82.

Woodford, N., Dallow, J. W. T., Hill, R. L. R., Palepou, M. F. I., Pike, R., Ward, M. E., Warner, M. & Livermore, D. M. (2007) Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *International Journal of Antimicrobial Agents* 29, 456-9.

Woodford, N., Ellington, M. J., Coelho, J. M., Turton, J. F., Ward, M. E., Brown, S., Amyes, S. G. B. & Livermore, D. M. (2006) Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *International Journal of Antimicrobial Agents* 27, 351-3.

Woodford, N. & Sundsfjord, A. (2005) Molecular detection of antibiotic resistance: when and where? *Journal of Antimicrobial Chemotherapy* 56, 259-61.

Woodford, N., Turton, J. F. & Livermore, D. M. (2011) Multiresistant Gram-negative bacteria: the role of high risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews* 35, 736-755.

Woodward, R., Yi, W., Li, L., Zhao, G., Eguchi, H., Perali, R. S., Guo, H., Song, J. J., Motari, E., Cai, L., Kelleher, P., Liu, X., Han, W., Zhang, W., Ding, Y., Li, M. & Wang, G. P. (2010) In vitro bacterial polysaccharide biosynthesis: defining the functions of Wzz and Wzy. *Nature Chemical Biology* 6, 418-423.

Wozniak, A., Undabarrena, A., Gallardo, N., Keller, N., Moraga, M., Mora, G. C. & Medicina, E. D. (2012) Porin alterations present in non carbapenemase-producing Enterobacteriaceae with high and intermediate-level of carbapenem resistance in Chile. *Journal of Medical Microbiology* 61, 1270-9.

Wu, S., Lourette, N. M., Tolic, N., Zhao, R., Robinson, E. W., Tolmachev, A. V., Smith, R. D. & Pasa-Tolic, L. (2009) An integrated top-down and bottom-up strategy for broadly characterizing protein isoforms and modifications. *Journal of Proteome Research* 8, 1347-1357.

Xu, C., Lin, X., Ren, H., Zhang, Y., Wang, S. & Peng, X. (2006) Analysis of outer membrane proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline. *Proteomics* 6, 462-73.

Yamaguchi, Y., Tomoyasu, T., Takaya, A., Morioka, M. & Yamamoto, T. (2003) Effects of disruption of heat shock genes on susceptibility of *Escherichia coli* to fluoroquinolones. *BMC Microbiology* 8, 1-8.

Yang, S., Lopez, C. R. & Zechiedrich, E. L. (2006) Quorum sensing and multidrug transporters in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 103, 2386-91.

Yang, S. Y. & Elzinga, M. (1993) Association of both enoyl coenzyme A hydratase and 3-hydroxyacyl coenzyme A epimerase with an active site in the amino-terminal domain of the multifunctional fatty acid oxidation protein from *Escherichia coli*. *Journal of Biological Chemistry* 268, 6588-6592.

Yates, J. R., Ruse, C. I., & Nakorchevsky, A. (2009) Proteomics by mass spectrometry: approaches, advances, and applications. *Annual Review of Biomedical Engineering* 11, 49-80.

Yeats, C. & Bateman, A. (2003) The BON domain: a putative membrane-binding domain. *Trends in Biochemical Sciences* 28, 352-355.

Yi, H., Cho, Y. J., Yong, D. & Chun, J. (2012) Genome sequence of *Escherichia coli* J53, a reference strain for genetic studies. *Journal of Bacteriology* 194, 3742-3743.

Yoshida, H., Maki, Y., Furuike, S., Sakai, A., Ueta, M. & Wada, A. (2012) YqjD is an inner membrane protein associated with stationary-phase ribosomes in *Escherichia coli*. *Journal of Bacteriology* 194, 4178-83.

Yun, S. H., Choi, C. W., Park, S. H., Lee, J. C., Leem, S. H., Choi, J. S., Kim, S. & Kim, S. I. (2008) Proteomic analysis of outer membrane proteins from *Acinetobacter baumannii* DU202 in tetracycline stress condition. *Journal of Microbiology* 46, 720-7.

Zavascki, A. P., Goldani, L. Z., Li, J. & Nation, R. L. (2007) Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of Antimicrobial Chemotherapy* 60, 1206-1215.

Zech, H., Echtermeyer, C., Wohlbrand, L., Blasius, B. & Rabus, R. (2011) Biological versus technical variability in 2-D DIGE experiments with environmental bacteria. *Proteomics* 11, 3380-3389.

Zhang, D. F., Jiang, B., Xiang, Z. M. & Wang, S. Y. (2008) Functional characterisation of altered outer membrane proteins for tetracycline resistance in *Escherichia coli*. *International Journal of Antimicrobial Agents* 32, 315-9.

Zhang, J., Mahdi, A. A., Briggs, G. S. & Lloyd, R. G. (2010) Promoting and avoiding recombination: contrasting activities of the *Escherichia coli* RuvABC holliday junction resolvase and RecG DNA translocase. *Genetics* 185, 23-37.

Zhang, W., Culley, D. E., Gritsenko, M. A., Moore, R. J., Camp, D. G., Smith, R. D. & Brockman, F. J. (2006) LC–MS/MS based proteomic analysis and functional inference of hypothetical proteins in *Desulfovibrio vulgaris*. *Biochemical and Biophysical Research Communications* 349, 1412-1419.

Zhou, H., Ning, Z., Starr, A. E., Abu-Farha, M. & Figeys, D. (2012) Advancements in top-down proteomics. *Analytical chemistry* 84, 720-734.

Zhou, L., Lei, X. H., Bochner, B. R. & Wanner, B. L. (2003) Phenotype MicroArray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *Journal of Bacteriology* 185, 4956-4972.

Zielke, R., Sikora, A. & Dutkiewicz, R. (2003) Involvement of the *cgtA* gene function in stimulation of DNA repair in *Escherichia coli* and *Vibrio harveyi*. *Microbiology* 149, 1763-1770.

Zijnge, V., Kieselbach, T. & Oscarsson, J. (2012) Proteomics of protein secretion by *Aggregatibacter actinomycetemcomitans*. *PloS one* 7, e41662.

Zimbler, D. L., Penwell, W. F., Gaddy, J. A., Menke, S. M., Tomaras, A. P., Connerly, P. L. & Actis, L. A. (2009) Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*. *Biometals* 22, 23-32.

Zolkiewski, M. (2006) A camel passes through the eye of a needle: protein unfolding activity of Clp ATPases. *Molecular Microbiology* 61, 1094-1100.

Appendix 1

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 I-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-D,L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

PM4A MicroPlate™ Phosphorus and Sulfur Sources

A1 Negative Control	A2 Phosphate	A3 Pyrophosphate	A4 Trimeta-phosphate	A5 Tripoly-phosphate	A6 Triethyl Phosphate	A7 Hypophosphite	A8 Adenosine- 2'-monophosphate	A9 Adenosine- 3'-monophosphate	A10 Adenosine- 5'-monophosphate	A11 Adenosine- 2',3'-cyclic monophosphate	A12 Adenosine- 3',5'-cyclic monophosphate
B1 Thiophosphate	B2 Dithiophosphate	B3 D,L-α-Glycerol Phosphate	B4 β-Glycerol Phosphate	B5 Carbamyl Phosphate	B6 D-2-Phospho-Glyceric Acid	B7 D-3-Phospho-Glyceric Acid	B8 Guanosine- 2'-monophosphate	B9 Guanosine- 3'-monophosphate	B10 Guanosine- 5'-monophosphate	B11 Guanosine- 2',3'-cyclic monophosphate	B12 Guanosine- 3',5'-cyclic monophosphate
C1 Phosphoenol Pyruvate	C2 Phospho-Glycolic Acid	C3 D-Glucose-1-Phosphate	C4 D-Glucose-6-Phosphate	C5 2-Deoxy-D-Glucose 6-Phosphate	C6 D-Glucosamine-6-Phosphate	C7 6-Phospho-Gluconic Acid	C8 Cytidine- 2'-monophosphate	C9 Cytidine- 3'-monophosphate	C10 Cytidine- 5'-monophosphate	C11 Cytidine- 2',3'-cyclic monophosphate	C12 Cytidine- 3',5'-cyclic monophosphate
D1 D-Mannose-1-Phosphate	D2 D-Mannose-6-Phosphate	D3 Cysteamine-S-Phosphate	D4 Phospho-L-Arginine	D5 O-Phospho-D-Serine	D6 O-Phospho-L-Serine	D7 O-Phospho-L-Threonine	D8 Uridine- 2'-monophosphate	D9 Uridine- 3'-monophosphate	D10 Uridine- 5'-monophosphate	D11 Uridine- 2',3'-cyclic monophosphate	D12 Uridine- 3',5'-cyclic monophosphate
E1 O-Phospho-D-Tyrosine	E2 O-Phospho-L-Tyrosine	E3 Phosphocreatine	E4 Phosphoryl Choline	E5 O-Phosphoryl-Ethanolamine	E6 Phosphono Acetic Acid	E7 2-Aminoethyl Phosphonic Acid	E8 Methylene Diphosphonic Acid	E9 Thymidine- 3'-monophosphate	E10 Thymidine- 5'-monophosphate	E11 Inositol Hexaphosphate	E12 Thymidine 3',5'- cyclic monophosphate
F1 Negative Control	F2 Sulfate	F3 Thiosulfate	F4 Tetrathionate	F5 Thiophosphate	F6 Dithiophosphate	F7 L-Cysteine	F8 D-Cysteine	F9 L-Cysteinyl-Glycine	F10 L-Cysteic Acid	F11 Cysteamine	F12 L-Cysteine Sulfinic Acid
G1 N-Acetyl-L-Cysteine	G2 S-Methyl-L-Cysteine	G3 Cystathionine	G4 Lanthionine	G5 Glutathione	G6 D,L-Ethionine	G7 L-Methionine	G8 D-Methionine	G9 Glycyl-L-Methionine	G10 N-Acetyl-D,L-Methionine	G11 L- Methionine Sulfoxide	G12 L-Methionine Sulfone
H1 L-Djenkolic Acid	H2 Thiourea	H3 1-Thio-β-D-Glucose	H4 D,L-Lipoamide	H5 Taurocholic Acid	H6 Taurine	H7 Hypotaurine	H8 p-Amino Benzene Sulfonic Acid	H9 Butane Sulfonic Acid	H10 2-Hydroxyethane Sulfonic Acid	H11 Methane Sulfonic Acid	H12 Tetramethylene Sulfone

PM5 MicroPlate™ Nutrient Supplements

A1 Negative Control	A2 Positive Control	A3 L-Alanine	A4 L-Arginine	A5 L-Asparagine	A6 L-Aspartic Acid	A7 L-Cysteine	A8 L-Glutamic Acid	A9 Adenosine-3',5'-cyclic monophosphate	A10 Adenine	A11 Adenosine	A12 2'-Deoxy Adenosine
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 Guanosine-3',5'-cyclic monophosphate	B10 Guanine	B11 Guanosine	B12 2'-Deoxy Guanosine
C1 L-Proline	C2 L-Serine	C3 L-Threonine	C4 L-Tryptophan	C5 L-Tyrosine	C6 L-Valine	C7 L-Isoleucine + L-Valine	C8 trans-4-Hydroxy L-Proline	C9 (5) 4-Amino-Imidazole-4(5)-Carboxamide	C10 Hypoxanthine	C11 Inosine	C12 2'-Deoxy Inosine
D1 L-Ornithine	D2 L-Citrulline	D3 Chorismic Acid	D4 (-)Shikimic Acid	D5 L-Homoserine Lactone	D6 D-Alanine	D7 D-Aspartic Acid	D8 D-Glutamic Acid	D9 D,L-α,ε-Diamino-pimelic Acid	D10 Cytosine	D11 Cytidine	D12 2'-Deoxy Cytidine
E1 Putrescine	E2 Spermidine	E3 Spermine	E4 Pyridoxine	E5 Pyridoxal	E6 Pyridoxamine	E7 β-Alanine	E8 D-Pantothenic Acid	E9 Orotic Acid	E10 Uracil	E11 Uridine	E12 2'-Deoxy Uridine
F1 Quinolinic Acid	F2 Nicotinic Acid	F3 Nicotinamide	F4 β-Nicotinamide Adenine Dinucleotide	F5 δ-Amino-Levulinic Acid	F6 Hematin	F7 Deferoxamine Mesylate	F8 D-(+)-Glucose	F9 N-Acetyl D-Glucosamine	F10 Thymine	F11 Glutathione (reduced form)	F12 Thymidine
G1 Oxaloacetic Acid	G2 D-Biotin	G3 Cyano-Cobalamine	G4 p-Amino-Benzoic Acid	G5 Folic Acid	G6 Inosine + Thiamine	G7 Thiamine	G8 Thiamine Pyrophosphate	G9 Riboflavin	G10 Pyrrolo-Quinoline Quinone	G11 Menadione	G12 Myo-Inositol
H1 Butyric Acid	H2 D,L-α-Hydroxy-Butyric Acid	H3 α-Ketobutyric Acid	H4 Caprylic Acid	H5 D,L-α-Lipoic Acid (oxidized form)	H6 D,L-Mevalonic Acid	H7 D,L-Carnitine	H8 Choline	H9 Tween 20	H10 Tween 40	H11 Tween 60	H12 Tween 80

PM6 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Ala	A4 Ala-Arg	A5 Ala-Asn	A6 Ala-Glu	A7 Ala-Gly	A8 Ala-His	A9 Ala-Leu	A10 Ala-Lys	A11 Ala-Phe	A12 Ala-Pro
B1 Ala-Ser	B2 Ala-Thr	B3 Ala-Trp	B4 Ala-Tyr	B5 Arg-Ala	B6 Arg-Arg	B7 Arg-Asp	B8 Arg-Gln	B9 Arg-Glu	B10 Arg-Ile	B11 Arg-Leu	B12 Arg-Lys
C1 Arg-Met	C2 Arg-Phe	C3 Arg-Ser	C4 Arg-Trp	C5 Arg-Tyr	C6 Arg-Val	C7 Asn-Glu	C8 Asn-Val	C9 Asp-Asp	C10 Asp-Glu	C11 Asp-Leu	C12 Asp-Lys
D1 Asp-Phe	D2 Asp-Trp	D3 Asp-Val	D4 Cys-Gly	D5 Gln-Gln	D6 Gln-Gly	D7 Glu-Asp	D8 Glu-Glu	D9 Glu-Gly	D10 Glu-Ser	D11 Glu-Trp	D12 Glu-Tyr
E1 Glu-Val	E2 Gly-Ala	E3 Gly-Arg	E4 Gly-Cys	E5 Gly-Gly	E6 Gly-His	E7 Gly-Leu	E8 Gly-Lys	E9 Gly-Met	E10 Gly-Phe	E11 Gly-Pro	E12 Gly-Ser
F1 Gly-Thr	F2 Gly-Trp	F3 Gly-Tyr	F4 Gly-Val	F5 His-Asp	F6 His-Gly	F7 His-Leu	F8 His-Lys	F9 His-Met	F10 His-Pro	F11 His-Ser	F12 His-Trp
G1 His-Tyr	G2 His-Val	G3 Ile-Ala	G4 Ile-Arg	G5 Ile-Gln	G6 Ile-Gly	G7 Ile-His	G8 Ile-Ile	G9 Ile-Met	G10 Ile-Phe	G11 Ile-Pro	G12 Ile-Ser
H1 Ile-Trp	H2 Ile-Tyr	H3 Ile-Val	H4 Leu-Ala	H5 Leu-Arg	H6 Leu-Asp	H7 Leu-Glu	H8 Leu-Gly	H9 Leu-Ile	H10 Leu-Leu	H11 Leu-Met	H12 Leu-Phe

PM7 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Leu-Ser	A4 Leu-Trp	A5 Leu-Val	A6 Lys-Ala	A7 Lys-Arg	A8 Lys-Glu	A9 Lys-Ile	A10 Lys-Leu	A11 Lys-Lys	A12 Lys-Phe
B1 Lys-Pro	B2 Lys-Ser	B3 Lys-Thr	B4 Lys-Trp	B5 Lys-Tyr	B6 Lys-Val	B7 Met-Arg	B8 Met-Asp	B9 Met-Gln	B10 Met-Glu	B11 Met-Gly	B12 Met-His
C1 Met-Ile	C2 Met-Leu	C3 Met-Lys	C4 Met-Met	C5 Met-Phe	C6 Met-Pro	C7 Met-Trp	C8 Met-Val	C9 Phe-Ala	C10 Phe-Gly	C11 Phe-Ile	C12 Phe-Phe
D1 Phe-Pro	D2 Phe-Ser	D3 Phe-Trp	D4 Pro-Ala	D5 Pro-Asp	D6 Pro-Gln	D7 Pro-Gly	D8 Pro-Hyp	D9 Pro-Leu	D10 Pro-Phe	D11 Pro-Pro	D12 Pro-Tyr
E1 Ser-Ala	E2 Ser-Gly	E3 Ser-His	E4 Ser-Leu	E5 Ser-Met	E6 Ser-Phe	E7 Ser-Pro	E8 Ser-Ser	E9 Ser-Tyr	E10 Ser-Val	E11 Thr-Ala	E12 Thr-Arg
F1 Thr-Glu	F2 Thr-Gly	F3 Thr-Leu	F4 Thr-Met	F5 Thr-Pro	F6 Trp-Ala	F7 Trp-Arg	F8 Trp-Asp	F9 Trp-Glu	F10 Trp-Gly	F11 Trp-Leu	F12 Trp-Lys
G1 Trp-Phe	G2 Trp-Ser	G3 Trp-Trp	G4 Trp-Tyr	G5 Tyr-Ala	G6 Tyr-Gln	G7 Tyr-Glu	G8 Tyr-Gly	G9 Tyr-His	G10 Tyr-Leu	G11 Tyr-Lys	G12 Tyr-Phe
H1 Tyr-Trp	H2 Tyr-Tyr	H3 Val-Arg	H4 Val-Asn	H5 Val-Asp	H6 Val-Gly	H7 Val-His	H8 Val-Ile	H9 Val-Leu	H10 Val-Tyr	H11 Val-Val	H12 Y-Glu-Gly

PM8 MicroPlate™ Peptide Nitrogen Sources

A1 Negative Control	A2 Positive Control: L-Glutamine	A3 Ala-Asp	A4 Ala-Gln	A5 Ala-Ile	A6 Ala-Met	A7 Ala-Val	A8 Asp-Ala	A9 Asp-Gln	A10 Asp-Gly	A11 Glu-Ala	A12 Gly-Asn
B1 Gly-Asp	B2 Gly-Ile	B3 His-Ala	B4 His-Glu	B5 His-His	B6 Ile-Asn	B7 Ile-Leu	B8 Leu-Asn	B9 Leu-His	B10 Leu-Pro	B11 Leu-Tyr	B12 Lys-Asp
C1 Lys-Gly	C2 Lys-Met	C3 Met-Thr	C4 Met-Tyr	C5 Phe-Asp	C6 Phe-Glu	C7 Gln-Glu	C8 Phe-Met	C9 Phe-Tyr	C10 Phe-Val	C11 Pro-Arg	C12 Pro-Asn
D1 Pro-Glu	D2 Pro-Ile	D3 Pro-Lys	D4 Pro-Ser	D5 Pro-Trp	D6 Pro-Val	D7 Ser-Asn	D8 Ser-Asp	D9 Ser-Gln	D10 Ser-Glu	D11 Thr-Asp	D12 Thr-Gln
E1 Thr-Phe	E2 Thr-Ser	E3 Trp-Val	E4 Tyr-Ile	E5 Tyr-Val	E6 Val-Ala	E7 Val-Gln	E8 Val-Glu	E9 Val-Lys	E10 Val-Met	E11 Val-Phe	E12 Val-Pro
F1 Val-Ser	F2 β-Ala-Ala	F3 β-Ala-Gly	F4 β-Ala-His	F5 Met-β-Ala	F6 β-Ala-Phe	F7 D-Ala-D-Ala	F8 D-Ala-Gly	F9 D-Ala-Leu	F10 D-Leu-D-Leu	F11 D-Leu-Gly	F12 D-Leu-Tyr
G1 γ-Glu-Gly	G2 γ-D-Glu-Gly	G3 Gly-D-Ala	G4 Gly-D-Asp	G5 Gly-D-Ser	G6 Gly-D-Thr	G7 Gly-D-Val	G8 Leu-β-Ala	G9 Leu-D-Leu	G10 Phe-β-Ala	G11 Ala-Ala-Ala	G12 D-Ala-Gly-Gly
H1 Gly-Gly-Ala	H2 Gly-Gly-D-Leu	H3 Gly-Gly-Gly	H4 Gly-Gly-Ile	H5 Gly-Gly-Leu	H6 Gly-Gly-Phe	H7 Val-Tyr-Val	H8 Gly-Phe-Phe	H9 Leu-Gly-Gly	H10 Leu-Leu-Leu	H11 Phe-Gly-Gly	H12 Tyr-Gly-Gly

PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 5.5%	A7 NaCl 6%	A8 NaCl 6.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N-N Dimethyl glycine	B4 NaCl 6% + Sarcosine	B5 NaCl 6% + Dimethyl sulphonyl propionate	B6 NaCl 6% + MOPS	B7 NaCl 6% + Ectoine	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + Creatinine	B12 NaCl 6% + L- Carnitine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-proline	C3 NaCl 6% + N-Acethyl L-glutamine	C4 NaC1 6% + β-Glutamic acid	C5 NaC1 6% + γ–Amino -n-butyric acid	C6 NaC1 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaC1 6% + Trehalose	C9 NaC1 6% + Trimethylamine -N-oxide	C10 NaC1 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaC1 6% + Trigonelline
D-1 Potassium chloride 3%	D2 Potassium chloride 4%	D3 Potassium chloride 5%	D4 Potassium chloride 6%	D5 Sodium sulfate 2%	D6 Sodium sulfate 3%	D7 Sodium sulfate 4%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 11%	F12 Sodium Lactate 12%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH8 10mM	G10 Ammonium sulfate pH 8 20mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrite 10mM	H8 Sodium Nitrite 20mM	H9 Sodium Nitrite 40mM	H10 Sodium Nitrite 60mM	H11 Sodium Nitrite 80mM	H12 Sodium Nitrite 100mM

PM10 MicroPlate™ pH

A1 pH 3.5	A2 pH 4	A3 pH 4.5	A4 pH 5	A5 pH 5.5	A6 pH 6	A7 pH 7	A8 pH 8	A9 pH 8.5	A10 pH 9	A11 pH 9.5	A12 pH 10
B1 pH 4.5	B2 pH 4.5 + L-Alanine	B3 pH 4.5 + L-Arginine	B4 pH 4.5 + L-Asparagine	B5 pH 4.5 + L-Aspartic Acid	B6 pH 4.5 + L-Glutamic Acid	B7 pH 4.5 + L-Glutamine	B8 pH 4.5 + Glycine	B9 pH 4.5 + L-Histidine	B10 pH 4.5 + L-Isoleucine	B11 pH 4.5 + L-Leucine	B12 pH 4.5 + L-Lysine
C1 pH 4.5 + L-Methionine	C2 pH 4.5 + L-Phenylalanine	C3 pH 4.5 + L-Proline	C4 pH 4.5 + L-Serine	C5 pH 4.5 + L-Threonine	C6 pH 4.5 + L-Tryptophan	C7 pH 4.5 + L-Tyrosine	C8 pH 4.5 + L-Valine	C9 pH 4.5 + Hydroxy-L-Proline	C10 pH 4.5 + L-Ornithine	C11 pH 4.5 + L-Homoarginine	C12 pH 4.5 + L-Homoserine
D-1 pH 4.5 + Anthranilic acid	D2 pH 4.5 + L-Norleucine	D3 pH 4.5 + L-Norvaline	D4 pH 4.5 + α- Amino-N-butyric acid	D5 pH 4.5 + p-Aminobenzoate	D6 pH 4.5 + L-Cysteic acid	D7 pH 4.5 + D-Lysine	D8 pH 4.5 + 5-Hydroxy Lysine	D9 pH 4.5 + 5-Hydroxy Tryptophan	D10 pH 4.5 + D,L-Diamino pimelic acid	D11 pH 4.5 + Trimethyl amine-N-oxide	D12 pH 4.5 + Urea
E1 pH 9.5	E2 pH 9.5 + L-Alanine	E3 pH 9.5 + L-Arginine	E4 pH 9.5 + L-Asparagine	E5 pH 9.5 + L-Aspartic Acid	E6 pH 9.5 + L-Glutamic Acid	E7 pH 9.5 + L-Glutamine	E8 pH 9.5 + Glycine	E9 pH 9.5 + L-Histidine	E10 pH 9.5 + L-Isoleucine	E11 pH 9.5 + L-Leucine	E12 pH 9.5 + L-Lysine
F1 pH 9.5 + L-Methionine	F2 pH 9.5 + L-Phenylalanine	F3 pH 9.5 + L-Proline	F4 pH 9.5 + L-Serine	F5 pH 9.5 + L-Threonine	F6 pH 9.5 + L-Tryptophan	F7 pH 9.5 + L-Tyrosine	F8 pH 9.5 + L-Valine	F9 pH 9.5 + Hydroxy-L-Proline	F10 pH 9.5 + L-Ornithine	F11 pH 9.5 + L-Homoarginine	F12 pH 9.5 + L-Homoserine
G1 pH 9.5 + Anthranilic acid	G2 pH 9.5 + L-Norleucine	G3 pH 9.5 + L-Norvaline	G4 pH 9.5 + Agmatine	G5 pH 9.5 + Cadaverine	G6 pH 9.5 + Putrescine	G7 pH 9.5 + Histamine	G8 pH 9.5 + Phenylethylamine	G9 pH 9.5 + Tyramine	G10 pH 9.5 + Creatine	G11 pH 9.5 + Trimethyl amine-N-oxide	G12 pH 9.5 + Urea
H1 X-Caprylate	H2 X-α-D-Glucoside	H3 X-β-D-Glucoside	H4 X-α-D-Galactoside	H5 X-β-D-Galactoside	H6 X-α- D-Glucuronide	H7 X-β- D-Glucuronide	H8 X-β-D-Glucosaminide	H9 X-β-D-Galactosaminide	H10 X-α-D-Mannoside	H11 X-PO4	H12 X-SO4

PM11C MicroPlate™

A1 Amikacin 1	A2 Amikacin 2	A3 Amikacin 3	A4 Amikacin 4	A5 Chlortetracycline 1	A6 Chlortetracycline 2	A7 Chlortetracycline 3	A8 Chlortetracycline 4	A9 Lincomycin 1	A10 Lincomycin 2	A11 Lincomycin 3	A12 Lincomycin 4
B1 Amoxicillin 1	B2 Amoxicillin 2	B3 Amoxicillin 3	B4 Amoxicillin 4	B5 Cloxacillin 1	B6 Cloxacillin 2	B7 Cloxacillin 3	B8 Cloxacillin 4	B9 Lomefloxacin 1	B10 Lomefloxacin 2	B11 Lomefloxacin 3	B12 Lomefloxacin 4
C1 Bleomycin 1	C2 Bleomycin 2	C3 Bleomycin 3	C4 Bleomycin 4	C5 Colistin 1	C6 Colistin 2	C7 Colistin 3	C8 Colistin 4	C9 Minocycline 1	C10 Minocycline 2	C11 Minocycline 3	C12 Minocycline 4
D1 Capreomycin 1	D2 Capreomycin 2	D3 Capreomycin 3	D4 Capreomycin 4	D5 Demeclocyline 1	D6 Demeclocyline 2	D7 Demeclocyline 3	D8 Demeclocyline 4	D9 Nafcillin 1	D10 Nafcillin 2	D11 Nafcillin 3	D12 Nafcillin 4
E1 Cefazolin 1	E2 Cefazolin 2	E3 Cefazolin 3	E4 Cefazolin 4	E5 Enoxacin 1	E6 Enoxacin 2	E7 Enoxacin 3	E8 Enoxacin 4	E9 Nalidixic acid 1	E10 Nalidixic acid 2	E11 Nalidixic acid 3	E12 Nalidixic acid 4
F1 Chloramphenicol 1	F2 Chloramphenicol 2	F3 Chloramphenicol 3	F4 Chloramphenicol 4	F5 Erythromycin 1	F6 Erythromycin 2	F7 Erythromycin 3	F8 Erythromycin 4	F9 Neomycin 1	F10 Neomycin 2	F11 Neomycin 3	F12 Neomycin 4
G1 Ceftriaxone 1	G2 Ceftriaxone 2	G3 Ceftriaxone 3	G4 Ceftriaxone 4	G5 Gentamicin 1	G6 Gentamicin 2	G7 Gentamicin 3	G8 Gentamicin 4	G9 Potassium tellurite 1	G10 Potassium tellurite 2	G11 Potassium tellurite 3	G12 Potassium tellurite 4
H1 Cephalothin 1	H2 Cephalothin 2	H3 Cephalothin 3	H4 Cephalothin 4	H5 Kanamycin 1	H6 Kanamycin 2	H7 Kanamycin 3	H8 Kanamycin 4	H9 Ofloxacin 1	H10 Ofloxacin 2	H11 Ofloxacin 3	H12 Ofloxacin 4

PM12B MicroPlate™

A1 Penicillin G 1	A2 Penicillin G 2	A3 Penicillin G 3	A4 Penicillin G 4	A5 Tetracycline 1	A6 Tetracycline 2	A7 Tetracycline 3	A8 Tetracycline 4	A9 Carbenicillin 1	A10 Carbenicillin 2	A11 Carbenicillin 3	A12 Carbenicillin 4
B1 Oxacillin 1	B2 Oxacillin 2	B3 Oxacillin 3	B4 Oxacillin 4	B5 Penimepicycline 1	B6 Penimepicycline 2	B7 Penimepicycline 3	B8 Penimepicycline 4	B9 Polymyxin B 1	B10 Polymyxin B 2	B11 Polymyxin B 3	B12 Polymyxin B 4
C1 Paromomycin 1	C2 Paromomycin 2	C3 Paromomycin 3	C4 Paromomycin 4	C5 Vancomycin 1	C6 Vancomycin 2	C7 Vancomycin 3	C8 Vancomycin 4	C9 D,L-Serine hydroxamate 1	C10 D,L-Serine hydroxamate 2	C11 D,L-Serine hydroxamate 3	C12 D,L-Serine hydroxamate 4
D1 Sisomicin 1	D2 Sisomicin 2	D3 Sisomicin 3	D4 Sisomicin 4	D5 Sulfamethazine 1	D6 Sulfamethazine 2	D7 Sulfamethazine 3	D8 Sulfamethazine 4	D9 Novobiocin 1	D10 Novobiocin 2	D11 Novobiocin 3	D12 Novobiocin 4
E1 2,4-Diamino-6,7-diisopropyl-pteridine 1	E2 2,4-Diamino-6,7-diisopropyl-pteridine 2	E3 2,4-Diamino-6,7-diisopropyl-pteridine 3	E4 2,4-Diamino-6,7-diisopropyl-pteridine 4	E5 Sulfadiazine 1	E6 Sulfadiazine 2	E7 Sulfadiazine 3	E8 Sulfadiazine 4	E9 Benzethonium chloride 1	E10 Benzethonium chloride 2	E11 Benzethonium chloride 3	E12 Benzethonium chloride 4
F1 Tobramycin 1	F2 Tobramycin 2	F3 Tobramycin 3	F4 Tobramycin 4	F5 Sulfathiazole 1	F6 Sulfathiazole 2	F7 Sulfathiazole 3	F8 Sulfathiazole 4	F9 5-Fluoroorotic acid 1	F10 5-Fluoroorotic acid 2	F11 5-Fluoroorotic acid 3	F12 5-Fluoroorotic acid 4
G1 Spectinomycin 1	G2 Spectinomycin 2	G3 Spectinomycin 3	G4 Spectinomycin 4	G5 Sulfamethoxazole 1	G6 Sulfamethoxazole 2	G7 Sulfamethoxazole 3	G8 Sulfamethoxazole 4	G9 L- Aspartic -β-hydroxamate 1	G10 L- Aspartic -β-hydroxamate 2	G11 L- Aspartic -β-hydroxamate 3	G12 L- Aspartic -β-hydroxamate 4
H1 Spiramycin 1	H2 Spiramycin 2	H3 Spiramycin 3	H4 Spiramycin 4	H5 Rifampicin 1	H6 Rifampicin 2	H7 Rifampicin 3	H8 Rifampicin 4	H9 Dodecyltrimethyl ammonium bromide 1	H10 Dodecyltrimethyl ammonium bromide 2	H11 Dodecyltrimethyl ammonium bromide 3	H12 Dodecyltrimethyl ammonium bromide 4

PM13B MicroPlate™

A1 Ampicillin 1	A2 Ampicillin 2	A3 Ampicillin 3	A4 Ampicillin 4	A5 Dequalinium chloride 1	A6 Dequalinium chloride 2	A7 Dequalinium chloride 3	A8 Dequalinium chloride 4	A9 Nickel chloride 1	A10 Nickel chloride 2	A11 Nickel chloride 3	A12 Nickel chloride 4
B1 Azlocillin 1	B2 Azlocillin 2	B3 Azlocillin 3	B4 Azlocillin 4	B5 2,2'-Dipyridyl 1	B6 2,2'-Dipyridyl 2	B7 2,2'-Dipyridyl 3	B8 2,2'-Dipyridyl 4	B9 Oxolinic acid 1	B10 Oxolinic acid 2	B11 Oxolinic acid 3	B12 Oxolinic acid 4
C1 6-Mercaptopurine 1	C2 6-Mercaptopurine 2	C3 6-Mercaptopurine 3	C4 6-Mercaptopurine 4	C5 Doxycycline 1	C6 Doxycycline 2	C7 Doxycycline 3	C8 Doxycycline 4	C9 Potassium chromate 1	C10 Potassium chromate 2	C11 Potassium chromate 3	C12 Potassium chromate 4
D1 Cefuroxime 1	D2 Cefuroxime 2	D3 Cefuroxime 3	D4 Cefuroxime 4	D5 5-Fluorouracil 1	D6 5-Fluorouracil 2	D7 5-Fluorouracil 3	D8 5-Fluorouracil 4	D9 Rolitetracycline 1	D10 Rolitetracycline 2	D11 Rolitetracycline 3	D12 Rolitetracycline 4
E1 Cytosine arabinoside 1	E2 Cytosine arabinoside 2	E3 Cytosine arabinoside 3	E4 Cytosine arabinoside 4	E5 Geneticin (G418) 1	E6 Geneticin (G418) 2	E7 Geneticin (G418) 3	E8 Geneticin (G418) 4	E9 Ruthenium red 1	E10 Ruthenium red 2	E11 Ruthenium red 3	E12 Ruthenium red 4
F1 Cesium chloride 1	F2 Cesium chloride 2	F3 Cesium chloride 3	F4 Cesium chloride 4	F5 Glycine 1	F6 Glycine 2	F7 Glycine 3	F8 Glycine 4	F9 Thallium (I) acetate 1	F10 Thallium (I) acetate 2	F11 Thallium (I) acetate 3	F12 Thallium (I) acetate 4
G1 Cobalt chloride 1	G2 Cobalt chloride 2	G3 Cobalt chloride 3	G4 Cobalt chloride 4	G5 Manganese (II) chloride 1	G6 Manganese (II) chloride 2	G7 Manganese (II) chloride 3	G8 Manganese (II) chloride 4	G9 Trifluoperazine 1	G10 Trifluoperazine 2	G11 Trifluoperazine 3	G12 Trifluoperazine 4
H1 Cupric chloride 1	H2 Cupric chloride 2	H3 Cupric chloride 3	H4 Cupric chloride 4	H5 Moxalactam 1	H6 Moxalactam 2	H7 Moxalactam 3	H8 Moxalactam 4	H9 Tylosin 1	H10 Tylosin 2	H11 Tylosin 3	H12 Tylosin 4

PM14A MicroPlate™

A1 Acriflavine 1	A2 Acriflavine 2	A3 Acriflavine 3	A4 Acriflavine 4	A5 Furaltadone 1	A6 Furaltadone 2	A7 Furaltadone 3	A8 Furaltadone 4	A9 Sanguinarine 1	A10 Sanguinarine 2	A11 Sanguinarine 3	A12 Sanguinarine 4
B1 9-Aminoacridine 1	B2 9-Aminoacridine 2	B3 9-Aminoacridine 3	B4 9-Aminoacridine 4	B5 Fusaric acid 1	B6 Fusaric acid 2	B7 Fusaric acid 3	B8 Fusaric acid 4	B9 Sodium arsenate 1	B10 Sodium arsenate 2	B11 Sodium arsenate 3	B12 Sodium arsenate 4
C1 Boric Acid 1	C2 Boric Acid 2	C3 Boric Acid 3	C4 Boric Acid 4	C5 1-Hydroxypyridine-2-thione 1	C6 1-Hydroxypyridine-2-thione 2	C7 1-Hydroxypyridine-2-thione 3	C8 1-Hydroxypyridine-2-thione 4	C9 Sodium cyanate 1	C10 Sodium cyanate 2	C11 Sodium cyanate 3	C12 Sodium cyanate 4
D1 Cadmium chloride 1	D2 Cadmium chloride 2	D3 Cadmium chloride 3	D4 Cadmium chloride 4	D5 Iodoacetate 1	D6 Iodoacetate 2	D7 Iodoacetate 3	D8 Iodoacetate 4	D9 Sodium dichromate 1	D10 Sodium dichromate 2	D11 Sodium dichromate 3	D12 Sodium dichromate 4
E1 Cefoxitin 1	E2 Cefoxitin 2	E3 Cefoxitin 3	E4 Cefoxitin 4	E5 Nitrofurantoin 1	E6 Nitrofurantoin 2	E7 Nitrofurantoin 3	E8 Nitrofurantoin 4	E9 Sodium metaborate 1	E10 Sodium metaborate 2	E11 Sodium metaborate 3	E12 Sodium metaborate 4
F1 Chloramphenicol 1	F2 Chloramphenicol 2	F3 Chloramphenicol 3	F4 Chloramphenicol 4	F5 Piperacillin 1	F6 Piperacillin 2	F7 Piperacillin 3	F8 Piperacillin 4	F9 Sodium metavanadate 1	F10 Sodium metavanadate 2	F11 Sodium metavanadate 3	F12 Sodium metavanadate 4
G1 Chelerythrine 1	G2 Chelerythrine 2	G3 Chelerythrine 3	G4 Chelerythrine 4	G5 Carbenicillin 1	G6 Carbenicillin 2	G7 Carbenicillin 3	G8 Carbenicillin 4	G9 Sodium Nitrite 1	G10 Sodium Nitrite 2	G11 Sodium Nitrite 3	G12 Sodium Nitrite 4
H1 EGTA 1	H2 EGTA 2	H3 EGTA 3	H4 EGTA 4	H5 Promethazine 1	H6 Promethazine 2	H7 Promethazine 3	H8 Promethazine 4	H9 Sodium orthovanadate 1	H10 Sodium orthovanadate 2	H11 Sodium orthovanadate 3	H12 Sodium orthovanadate 4

PM15B MicroPlate™

A1 Procaine 1	A2 Procaine 2	A3 Procaine 3	A4 Procaine 4	A5 Guanidine hydrochloride 1	A6 Guanidine hydrochloride 2	A7 Guanidine hydrochloride 3	A8 Guanidine hydrochloride 4	A9 Cefmetazole 1	A10 Cefmetazole 2	A11 Cefmetazole 3	A12 Cefmetazole 4
B1 D-Cycloserine 1	B2 D-Cycloserine 2	B3 D-Cycloserine 3	B4 D-Cycloserine 4	B5 EDTA 1	B6 EDTA 2	B7 EDTA 3	B8 EDTA 4	B9 5,7-Dichloro-8-hydroxy-quinaldine 1	B10 5,7-Dichloro-8-hydroxy-quinaldine 2	B11 5,7-Dichloro-8-hydroxy-quinaldine 3	B12 5,7-Dichloro-8-hydroxy-quinaldine 4
C1 5,7-Dichloro-8-hydroxyquinoline 1	C2 5,7-Dichloro-8-hydroxyquinoline 2	C3 5,7-Dichloro-8-hydroxyquinoline 3	C4 5,7-Dichloro-8-hydroxyquinoline 4	C5 Fusidic acid 1	C6 Fusidic acid 2	C7 Fusidic acid 3	C8 Fusidic acid 4	C9 1,10-Phenanthroline 1	C10 1,10-Phenanthroline 2	C11 1,10-Phenanthroline 3	C12 1,10-Phenanthroline 4
D1 Phleomycin 1	D2 Phleomycin 2	D3 Phleomycin 3	D4 Phleomycin 4	D5 Domiphen bromide 1	D6 Domiphen bromide 2	D7 Domiphen bromide 3	D8 Domiphen bromide 4	D9 Nordihydroguaia retic acid 1	D10 Nordihydroguaia retic acid 2	D11 Nordihydroguaia retic acid 3	D12 Nordihydroguaia retic acid 4
E1 Alexidine 1	E2 Alexidine 2	E3 Alexidine 3	E4 Alexidine 4	E5 Nitrofurazone 1	E6 Nitrofurazone 2	E7 Nitrofurazone 3	E8 Nitrofurazone 4	E9 Methyl viologen 1	E10 Methyl viologen 2	E11 Methyl viologen 3	E12 Methyl viologen 4
F1 3, 4-Dimethoxybenzyl alcohol 1	F2 3, 4-Dimethoxybenzyl alcohol 2	F3 3, 4-Dimethoxybenzyl alcohol 3	F4 3, 4-Dimethoxybenzyl alcohol 4	F5 Oleandomycin 1	F6 Oleandomycin 2	F7 Oleandomycin 3	F8 Oleandomycin 4	F9 Puromycin 1	F10 Puromycin 2	F11 Puromycin 3	F12 Puromycin 4
G1 CCCP 1	G2 CCCP 2	G3 CCCP 3	G4 CCCP 4	G5 Sodium azide 1	G6 Sodium azide 2	G7 Sodium azide 3	G8 Sodium azide 4	G9 Menadione 1	G10 Menadione 2	G11 Menadione 3	G12 Menadione 4
H1 2-Nitroimidazole 1	H2 2-Nitroimidazole 2	H3 2-Nitroimidazole 3	H4 2-Nitroimidazole 4	H5 Hydroxyurea 1	H6 Hydroxyurea 2	H7 Hydroxyurea 3	H8 Hydroxyurea 4	H9 Zinc chloride 1	H10 Zinc chloride 2	H11 Zinc chloride 3	H12 Zinc chloride 4

PM16A MicroPlate™

A1 Cefotaxime 1	A2 Cefotaxime 2	A3 Cefotaxime 3	A4 Cefotaxime 4	A5 Phosphomycin 1	A6 Phosphomycin 2	A7 Phosphomycin 3	A8 Phosphomycin 4	A9 5-Chloro-7-iodo-8-hydroxyquinoline 1	A10 5-Chloro-7-iodo-8-hydroxyquinoline 2	A11 5-Chloro-7-iodo-8-hydroxyquinoline 3	A12 5-Chloro-7-iodo-8-hydroxyquinoline 4
B1 Norfloxacin 1	B2 Norfloxacin 2	B3 Norfloxacin 3	B4 Norfloxacin 4	B5 Sulfanilamide 1	B6 Sulfanilamide 2	B7 Sulfanilamide 3	B8 Sulfanilamide 4	B9 Trimethoprim 1	B10 Trimethoprim 2	B11 Trimethoprim 3	B12 Trimethoprim 4
C1 Dichlofluanid 1	C2 Dichlofluanid 2	C3 Dichlofluanid 3	C4 Dichlofluanid 4	C5 Protamine sulfate 1	C6 Protamine sulfate 2	C7 Protamine sulfate 3	C8 Protamine sulfate 4	C9 Cetylpyridinium chloride 1	C10 Cetylpyridinium chloride 2	C11 Cetylpyridinium chloride 3	C12 Cetylpyridinium chloride 4
D1 1-Chloro-2,4-dinitrobenzene 1	D2 1-Chloro-2,4-dinitrobenzene 2	D3 1-Chloro-2,4-dinitrobenzene 3	D4 1-Chloro-2,4-dinitrobenzene 4	D5 Diamide 1	D6 Diamide 2	D7 Diamide 3	D8 Diamide 4	D9 Cinoxacin 1	D10 Cinoxacin 2	D11 Cinoxacin 3	D12 Cinoxacin 4
E1 Streptomycin 1	E2 Streptomycin 2	E3 Streptomycin 3	E4 Streptomycin 4	E5 5-Azacytidine 1	E6 5-Azacytidine 2	E7 5-Azacytidine 3	E8 5-Azacytidine 4	E9 Rifamycin SV 1	E10 Rifamycin SV 2	E11 Rifamycin SV 3	E12 Rifamycin SV 4
F1 Potassium tellurite 1	F2 Potassium tellurite 2	F3 Potassium tellurite 3	F4 Potassium tellurite 4	F5 Sodium selenite 1	F6 Sodium selenite 2	F7 Sodium selenite 3	F8 Sodium selenite 4	F9 Aluminum sulfate 1	F10 Aluminum sulfate 2	F11 Aluminum sulfate 3	F12 Aluminum sulfate 4
G1 Chromium chloride 1	G2 Chromium chloride 2	G3 Chromium chloride 3	G4 Chromium chloride 4	G5 Ferric chloride 1	G6 Ferric chloride 2	G7 Ferric chloride 3	G8 Ferric chloride 4	G9 L-Glutamic acid g-hydroxamate 1	G10 L-Glutamic acid g-hydroxamate 2	G11 L-Glutamic acid g-hydroxamate 3	G12 L-Glutamic acid g-hydroxamate 4
H1 Glycine hydroxamate 1	H2 Glycine hydroxamate 2	H3 Glycine hydroxamate 3	H4 Glycine hydroxamate 4	H5 Chloroxylenol 1	H6 Chloroxylenol 2	H7 Chloroxylenol 3	H8 Chloroxylenol 4	H9 Sorbic Acid 1	H10 Sorbic Acid 2	H11 Sorbic Acid 3	H12 Sorbic Acid 4

PM17A MicroPlate™

A1 D-Serine 1	A2 D-Serine 2	A3 D-Serine 3	A4 D-Serine 4	A5 β-Chloro-L-alanine 1	A6 β-Chloro-L-alanine 2	A7 β-Chloro-L-alanine 3	A8 β-Chloro-L-alanine 4	A9 Thiosalicylate 1	A10 Thiosalicylate 2	A11 Thiosalicylate 3	A12 Thiosalicylate 4
B1 Sodium salicylate 1	B2 Sodium salicylate 2	B3 Sodium salicylate 3	B4 Sodium salicylate 4	B5 Hygromycin B 1	B6 Hygromycin B 2	B7 Hygromycin B 3	B8 Hygromycin B 4	B9 Ethionamide 1	B10 Ethionamide 2	B11 Ethionamide 3	B12 Ethionamide 4
C1 4-Aminopyridine 1	C2 4-Aminopyridine 2	C3 4-Aminopyridine 3	C4 4-Aminopyridine 4	C5 Sulfachloro-pyridazine 1	C6 Sulfachloro-pyridazine 2	C7 Sulfachloro-pyridazine 3	C8 Sulfachloro-pyridazine 4	C9 Sulfamono-methoxine 1	C10 Sulfamono-methoxine 2	C11 Sulfamono-methoxine 3	C12 Sulfamono-methoxine 4
D1 Oxycarboxin 1	D2 Oxycarboxin 2	D3 Oxycarboxin 3	D4 Oxycarboxin 4	D5 Aminotriazole 1	D6 Aminotriazole 2	D7 Aminotriazole 3	D8 Aminotriazole 4	D9 Chlorpromazine 1	D10 Chlorpromazine 2	D11 Chlorpromazine 3	D12 Chlorpromazine 4
E1 Niaproof 1	E2 Niaproof 2	E3 Niaproof 3	E4 Niaproof 4	E5 Compound 48/80 1	E6 Compound 48/80 2	E7 Compound 48/80 3	E8 Compound 48/80 4	E9 Sodium tungstate 1	E10 Sodium tungstate 2	E11 Sodium tungstate 3	E12 Sodium tungstate 4
F1 Lithium chloride 1	F2 Lithium chloride 2	F3 Lithium chloride 3	F4 Lithium chloride 4	F5 D,L-Methionine hydroxamate 1	F6 D,L-Methionine hydroxamate 2	F7 D,L-Methionine hydroxamate 3	F8 D,L-Methionine hydroxamate 4	F9 Tannic acid 1	F10 Tannic acid 2	F11 Tannic acid 3	F12 Tannic acid 4
G1 Chlorambucil 1	G2 Chlorambucil 2	G3 Chlorambucil 3	G4 Chlorambucil 4	G5 Cefamandole 1	G6 Cefamandole 2	G7 Cefamandole 3	G8 Cefamandole 4	G9 Cefoperazone 1	G10 Cefoperazone 2	G11 Cefoperazone 3	G12 Cefoperazone 4
H1 Cefsulodin 1	H2 Cefsulodin 2	H3 Cefsulodin 3	H4 Cefsulodin 4	H5 Caffeine 1	H6 Caffeine 2	H7 Caffeine 3	H8 Caffeine 4	H9 Phenylarsine oxide 1	H10 Phenylarsine oxide 2	H11 Phenylarsine oxide 3	H12 Phenylarsine oxide 4

PM18C MicroPlate™

A1 Ketoprofen 1	A2 Ketoprofen 2	A3 Ketoprofen 3	A4 Ketoprofen 4	A5 Pyrophosphate 1	A6 Pyrophosphate 2	A7 Pyrophosphate 3	A8 Pyrophosphate 4	A9 Thiamphenicol 1	A10 Thiamphenicol 2	A11 Thiamphenicol 3	A12 Thiamphenicol 4
B1 Trifluorothymidine 1	B2 Trifluorothymidine 2	B3 Trifluorothymidine 3	B4 Trifluorothymidine 4	B5 Pipemidic Acid 1	B6 Pipemidic Acid 2	B7 Pipemidic Acid 3	B8 Pipemidic Acid 4	B9 Azathioprine 1	B10 Azathioprine 2	B11 Azathioprine 3	B12 Azathioprine 4
C1 Poly-L-lysine 1	C2 Poly-L-lysine 2	C3 Poly-L-lysine 3	C4 Poly-L-lysine 4	C5 Sulfisoxazole 1	C6 Sulfisoxazole 2	C7 Sulfisoxazole 3	C8 Sulfisoxazole 4	C9 Pentachlorophenol 1	C10 Pentachlorophenol 2	C11 Pentachlorophenol 3	C12 Pentachlorophenol 4
D1 Sodium m-arsenite 1	D2 Sodium m-arsenite 2	D3 Sodium m-arsenite 3	D4 Sodium m-arsenite 4	D5 Sodium bromate 1	D6 Sodium bromate 2	D7 Sodium bromate 3	D8 Sodium bromate 4	D9 Lidocaine 1	D10 Lidocaine 2	D11 Lidocaine 3	D12 Lidocaine 4
E1 Sodium metasilicate 1	E2 Sodium metasilicate 2	E3 Sodium metasilicate 3	E4 Sodium metasilicate 4	E5 Sodium periodate 1	E6 Sodium periodate 2	E7 Sodium periodate 3	E8 Sodium periodate 4	E9 Antimony (III) chloride 1	E10 Antimony (III) chloride 2	E11 Antimony (III) chloride 3	E12 Antimony (III) chloride 4
F1 Semicarbazide hydrochloride 1	F2 Semicarbazide hydrochloride 2	F3 Semicarbazide hydrochloride 3	F4 Semicarbazide hydrochloride 4	F5 Tinidazole 1	F6 Tinidazole 2	F7 Tinidazole 3	F8 Tinidazole 4	F9 Aztreonam 1	F10 Aztreonam 2	F11 Aztreonam 3	F12 Aztreonam 4
G1 Triclosan 1	G2 Triclosan 2	G3 Triclosan 3	G4 Triclosan 4	G5 Guanazole 1	G6 Guanazole 2	G7 Guanazole 3	G8 Guanazole 4	G9 Myricetin 1	G10 Myricetin 2	G11 Myricetin 3	G12 Myricetin 4
H1 5-Fluoro-5'-deoxyuridine 1	H2 5-Fluoro-5'-deoxyuridine 2	H3 5-Fluoro-5'-deoxyuridine 3	H4 5-Fluoro-5'-deoxyuridine 4	H5 2-Phenylphenol 1	H6 2-Phenylphenol 2	H7 2-Phenylphenol 3	H8 2-Phenylphenol 4	H9 Plumbagin 1	H10 Plumbagin 2	H11 Plumbagin 3	H12 Plumbagin 4

PM19 MicroPlate™

A1 Josamycin 1	A2 Josamycin 2	A3 Josamycin 3	A4 Josamycin 4	A5 Gallic acid 1	A6 Gallic acid 2	A7 Gallic acid 3	A8 Gallic acid 4	A9 Coumarin 1	A10 Coumarin 2	A11 Coumarin 3	A12 Coumarin 4
B1 Methyltrioctyl-ammonium chloride 1	B2 Methyltrioctyl-ammonium chloride 2	B3 Methyltrioctyl-ammonium chloride 3	B4 Methyltrioctyl-ammonium chloride 4	B5 Harmane 1	B6 Harmane 2	B7 Harmane 3	B8 Harmane 4	B9 2,4-Dinitrophenol 1	B10 2,4-Dinitrophenol 2	B11 2,4-Dinitrophenol 3	B12 2,4-Dinitrophenol 4
C1 Chlorhexidine 1	C2 Chlorhexidine 2	C3 Chlorhexidine 3	C4 Chlorhexidine 4	C5 Umbelliferone 1	C6 Umbelliferone 2	C7 Umbelliferone 3	C8 Umbelliferone 4	C9 Cinnamic acid 1	C10 Cinnamic acid 2	C11 Cinnamic acid 3	C12 Cinnamic acid 4
D1 Disulfiram 1	D2 Disulfiram 2	D3 Disulfiram 3	D4 Disulfiram 4	D5 Iodonitro tetrazolium violet 1	D6 Iodonitro tetrazolium violet 2	D7 Iodonitro tetrazolium violet 3	D8 Iodonitro tetrazolium violet 4	D9 Phenyl-methylsulfonyl-fluoride (PMSF) 1	D10 Phenyl-methylsulfonyl-fluoride (PMSF) 2	D11 Phenyl-methylsulfonyl-fluoride (PMSF) 3	D12 Phenyl-methylsulfonyl-fluoride (PMSF) 4
E1 FCCP 1	E2 FCCP 2	E3 FCCP 3	E4 FCCP 4	E5 D,L-Thioctic acid 1	E6 D,L-Thioctic acid 2	E7 D,L-Thioctic acid 3	E8 D,L-Thioctic acid 4	E9 Lawsone 1	E10 Lawsone 2	E11 Lawsone 3	E12 Lawsone 4
F1 Phenethicillin 1	F2 Phenethicillin 2	F3 Phenethicillin 3	F4 Phenethicillin 4	F5 Blasticidin S 1	F6 Blasticidin S 2	F7 Blasticidin S 3	F8 Blasticidin S 4	F9 Sodium caprylate 1	F10 Sodium caprylate 2	F11 Sodium caprylate 3	F12 Sodium caprylate 4
G1 Lauryl sulfobetaine 1	G2 Lauryl sulfobetaine 2	G3 Lauryl sulfobetaine 3	G4 Lauryl sulfobetaine 4	G5 Dihydro-streptomycin 1	G6 Dihydro-streptomycin 2	G7 Dihydro-streptomycin 3	G8 Dihydro-streptomycin 4	G9 Hydroxylamine 1	G10 Hydroxylamine 2	G11 Hydroxylamine 3	G12 Hydroxylamine 4
H1 Hexamminecobalt (III) Chloride 1	H2 Hexamminecobalt (III) Chloride 2	H3 Hexamminecobalt (III) Chloride 3	H4 Hexamminecobalt (III) Chloride 4	H5 Thioglycerol 1	H6 Thioglycerol 2	H7 Thioglycerol 3	H8 Thioglycerol 4	H9 Polymyxin B 1	H10 Polymyxin B 2	H11 Polymyxin B 3	H12 Polymyxin B 4

PM20B MicroPlate™

A1 Amitriptyline 1	A2 Amitriptyline 2	A3 Amitriptyline 3	A4 Amitriptyline 4	A5 Apramycin 1	A6 Apramycin 2	A7 Apramycin 3	A8 Apramycin 4	A9 Benserazide 1	A10 Benserazide 2	A11 Benserazide 3	A12 Benserazide 4
B1 Orphenadrine 1	B2 Orphenadrine 2	B3 Orphenadrine 3	B4 Orphenadrine 4	B5 Propranolol 1	B6 Propranolol 2	B7 Propranolol 3	B8 Propranolol 4	B9 Tetrazolium violet 1	B10 Tetrazolium violet 2	B11 Tetrazolium violet 3	B12 Tetrazolium violet 4
C1 Thioridazine 1	C2 Thioridazine 2	C3 Thioridazine 3	C4 Thioridazine 4	C5 Atropine 1	C6 Atropine 2	C7 Atropine 3	C8 Atropine 4	C9 Ornidazole 1	C10 Ornidazole 2	C11 Ornidazole 3	C12 Ornidazole 4
D1 Proflavine 1	D2 Proflavine 2	D3 Proflavine 3	D4 Proflavine 4	D5 Ciprofloxacin 1	D6 Ciprofloxacin 2	D7 Ciprofloxacin 3	D8 Ciprofloxacin 4	D9 18-Crown-6 ether 1	D10 18-Crown-6 ether 2	D11 18-Crown-6 ether 3	D12 18-Crown-6 ether 4
E1 Crystal Violet 1	E2 Crystal Violet 2	E3 Crystal Violet 3	E4 Crystal Violet 4	E5 Dodine 1	E6 Dodine 2	E7 Dodine 3	E8 Dodine 4	E9 Hexachlorophene 1	E10 Hexachlorophene 2	E11 Hexachlorophene 3	E12 Hexachlorophene 4
F1 4-Hydroxycoumarin 1	F2 4-Hydroxycoumarin 2	F3 4-Hydroxycoumarin 3	F4 4-Hydroxycoumarin 4	F5 Oxytetracycline 1	F6 Oxytetracycline 2	F7 Oxytetracycline 3	F8 Oxytetracycline 4	F9 Pridinol 1	F10 Pridinol 2	F11 Pridinol 3	F12 Pridinol 4
G1 Captan 1	G2 Captan 2	G3 Captan 3	G4 Captan 4	G5 3,5-Dinitrobenzene 1	G6 3,5-Dinitrobenzene 2	G7 3,5-Dinitrobenzene 3	G8 3,5-Dinitrobenzene 4	G9 8-Hydroxyquinoline 1	G10 8-Hydroxyquinoline 2	G11 8-Hydroxyquinoline 3	G12 8-Hydroxyquinoline 4
H1 Patulin 1	H2 Patulin 2	H3 Patulin 3	H4 Patulin 4	H5 Tolylfluanid 1	H6 Tolylfluanid 2	H7 Tolylfluanid 3	H8 Tolylfluanid 4	H9 Troleandomycin 1	H10 Troleandomycin 2	H11 Troleandomycin 3	H12 Troleandomycin 4